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JCS49 U.S. PTO

(Only for new nonprovisional applications under
37 C.F.R. 1.53(b))

HU98-02pA

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EL29099350145

OLIGOMERIZATION OF HEPATITIS DELTA ANTIGEN

ADDRESS TO:

Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

- Microfiche Computer Program (*Appendix*)

7. ☐ Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)

- a. ☐ Computer Readable Copy
- b. ☐ Paper Copy (identical to computer copy)
- c. ☐ Statement verifying identity of above copies

- 8 ☐ Assignment Papers (cover sheet & documents)

9. ☐ 37 C F R 3.73(b) Statement ☐ Power of Attorney
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- 10 ☐ English Translation Document (if applicable)

11. ☐ Information Disclosure Statement (IDS)/PTO-1449 ☐ Copies of IDS Citations

- 12 ☐ Preliminary Amendment

13. ☒ Return Receipt Postcard (MPEP 503) (2)
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14. ☐ Small Entity Statement(s) ☐ Statement filed in prior application, Status still proper and desired

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Under 37CFR 1.84(a)(2) and Amendment
.....and Transmittal of Formal Drawings

- Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application No. /

Prior application information: Examiner: _____ Group Art Unit: _____

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July 6, 1998

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42,122

Date: 7/1/99 Express Mail Label No. EL290993501 US

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Attorney's Docket No.: HU98-02p

OLIGOMERIZATION OF HEPATITIS DELTA ANTIGEN

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 60/091,609
filed July 2, 1998, the contents of which are incorporated herein by reference in their
5 entirety.

GOVERNMENT SUPPORT

The invention was made with Government support from the National Institutes
of Health under grant AI32480 and R01-HL37974 and the U.S. Public Health Service
(GM42031) and the Giovanni Armoris Harvard Center for Structural Biology. The
10 Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The hepatitis D virus (HDV) is a small satellite virus of hepatitis B virus (HBV).
Coinfection with HBV and HDV causes severe and sometimes fatal liver disease in
humans. The HDV genome encodes a single known protein, the hepatitis delta antigen
15 (HDAg).

SUMMARY OF THE INVENTION

This invention is based on the discovery of the high resolution crystal structure
of a synthetic peptide corresponding to residues 12-60 of the hepatitis delta antigen
20 (HDAg). This peptide includes a coiled-coil region believed to be important for

dimerization of HDAG. The peptide forms an antiparallel coiled coil with hydrophobic residues near the termini of each peptide forming an extensive hydrophobic core with residues C-terminal to the coiled-coil domain in the dimer protein. The crystal structure shows how HDAG forms dimers, but, surprisingly, also shows the dimers forming an
5 octameric structure that forms a large 50Å ring lined with basic sidechains.

The dimers associate further to form octamers through residues in the coiled-coil domain that are not involved in a heptad repeat, as well as through residues C-terminal to this region. The crystal structure of the peptide and cross-linking hydrodynamic studies which show that the full-length recombinant protein also forms octamers suggest
10 that the structure of the delta antigen represents a previously unseen organization of a viral nucleocapsid protein. This N-terminal octamer can serve as a convenient high-valency framework for linking a variety of functional peptides and domains.

The invention includes HDAG proteins, including derivatives, mutants and fragments, and nucleic acid molecules encoding HDAG. Derivatives of HDAG protein
15 include fusion molecules. In one embodiment, the fusion molecule comprises HDAG and at least one binding moiety bound, for example, to the HDAG through the C terminus, N terminus and/or other amino acid. The binding moiety can be selected from the group consisting of an antigen, an antibody, a ligand, a receptor, an enzyme, a ligand interaction peptide, a chemical, an effector, an oligonucleotide, a signal amplification
20 peptide, an enhancer recognition protein, a promoter binding protein, a label, a growth factor, a cytokine, a nuclease, a small organic molecule, a test substance, a cytotoxic agent, a substrate, a solid substrate, a drug, or a fragment thereof. The fusion molecules of the invention can also comprise two binding moieties which are binding partners. The fusion molecule can be a fusion protein. The HDAG and the binding moiety can be
25 chemically linked or the HDAG and the binding moiety can be expressed as a single unit.

The invention also relates to coiled-coil oligomers comprising at least two such fusion molecules. The coiled-coil oligomer can be an octamer. In the coiled-coil oligomer, the two fusion molecules can be the same or different.

The invention also relates to nucleic acid molecules. For example, a nucleic acid molecule can comprise a nucleotide sequence depicted in Figure 9, nucleotides 37 - 150 of Figure 9, nucleotides 37 - 186 of Figure 9, Figure 10, nucleotides 1421 - 1566 of Figure 10, nucleotides 1457 - 1566 of Figure 10, Figure 15 or Figure 16. The nucleic acid molecule can also comprise a nucleotide sequence which encodes a polypeptide comprising an amino acid sequence depicted in a row of Figure 1, amino acids 12 - 48 of a row of Figure 1, the top row of Figure 3C, Figure 9, amino acids 12 - 48 of a row of Figure 9, Figure 10, amino acids 12 - 88 of Figure 10, Figure 11 or Figure 17. Also included are complementary strands of these sequences, DNA sequences that hybridize to the sequences, RNA sequences transcribed from the sequences, or a fragment or mutation thereof, which encodes a coiled-coil oligomer.

15 An isolated nucleic acid molecule can be a fusion molecule described herein. The invention also includes fusion genes comprising an HDAG nucleic acid molecule operably linked to a nucleic acid molecule encoding a heterologous (non-HDAG) peptide.

Also encompassed in the scope of the invention are isolated, purified and/or recombinant peptides and molecules comprising peptides. In one embodiment, a polypeptide comprises an amino acid sequence encoded by an HDAG nucleic acid molecule. The molecules can comprise a polypeptide having an amino acid sequence selected from the group consisting of an amino acid sequence depicted in a row of Figure 1, amino acids 12 - 48 of a row of Figure 1, amino acids 12 - 60 of a row of Figure 1, the top row of Figure 3C, Figure 9, amino acids 12 - 48 of Figure 9, amino acids 12 - 60 of Figure 9, Figure 10, Figure 11 and Figure 17, or a fragment or derivative thereof which forms a coiled-coil oligomer. The peptide can be a derivative peptide wherein a serine residue is substituted with cysteine. The molecules can

comprise a polypeptide comprising an amino acid sequence of amino acids 12 - 88 of HDAG, or a fragment or derivative thereof which forms a coiled-coil oligomer and nuclear localization signal. The polypeptides can be encoded by fusion genes comprising HDAG. It is possible that the molecule can be larger than the 12-48 or 12-60 or 12-88 amino acids, for example. It may be desirable to make a 12-65 or 10-93 peptide, for example.

The invention also includes vectors which can express HDAG. The vectors can comprise a nucleic acid molecule which encodes a subunit of an HDAG coiled-coil octamer. The nucleic acid molecule can comprise a sequence listed above. The nucleic acid molecule can encode a fusion molecule. The vector can be a nucleic acid molecule encoding HDAG and at least one multiple cloning site. The multiple cloning site(s) can be located 3' to the nucleic acid molecule encoding HDAG or 5' to the nucleic acid molecule encoding HDAG. There can be two or more multiple coding sites, wherein at least one multiple coding site is located in a flanking region 3' to the nucleic acid molecular encoding HDAG and/or at least one multiple coding site is located in a flanking region 5' to the nucleic acid molecule encoding HDAG. The vector can further comprise a nucleic acid molecule encoding a nuclear localization signal. A vector can further comprise a nucleic acid molecule which encodes a heterologous gene. The vector can express a fusion molecule of HDAG wherein a first heterologous gene encodes a first binding moiety and a second heterologous gene encodes a second binding moiety.

The invention also encompasses host cells which comprise a nucleic acid molecule which encodes a molecule of HDAG, including a fusion molecule.

The invention also includes methods of manufacturing such a host cell comprising a nucleic acid molecule encoding a fusion molecule comprising HDAG and at least one binding moiety, by introducing a vector of the invention into the host cell.

The invention also relates to methods of using the molecules, i.e., peptides, nucleic acids, and vectors of the invention. One method comprises expressing a high

valency display of at least one binding moiety comprising introducing into a cell with a vector comprising a nucleic acid molecule encoding HDAG and a nucleic acid molecule encoding the binding entity and culturing the cell under conditions sufficient to permit expression of the binding moiety and HDAG.

- 5 The invention also encompasses a method of enhancing interaction between binding partners comprising contacting a fusion molecule of HDAG with a second binding moiety wherein the first and second moieties are binding partners. The fusion molecule can present the first and second moieties. The interaction between ligands can occur in solution, on membranes or on surfaces. The fusion molecule can be a subunit
- 10 of a coiled-coil oligomer, e.g., an octamer, and the first and second moieties are bound to the oligomer. In one embodiment, fusion of a first cell and a second cell is enhanced.

- The invention also includes a method for delivering molecules to a cell comprising contacting them with an HDAG fusion molecule. In one embodiment, the binding moiety is an oligonucleotide. The oligonucleotide can hybridize to a nucleic
- 15 acid molecule in the cell. The fusion molecule can further comprise a double-stranded nuclease. In one embodiment, the fusion molecule comprises a first binding moiety and a second binding moiety wherein the first binding moiety interacts with a binding partner and the second binding moiety functions as an effector. The first binding moiety can interact with a cell surface receptor on a cell and the second binding moiety
- 20 can kill the cell.

- The invention also includes a method of amplifying a signal in a solid phase assay comprising coupling an HDAG octamer with at least one copy of a domain which interacts with a ligand and at least two copies of a label. The label can be, for example, alkaline phosphatase, a radiolabel, streptadavin, and green fluorescent protein. In one
- 25 embodiment, the solid phase assay is an ELISA assay. The invention also encompasses a method of facilitating exchange of substrates and products comprising coupling an HDAG oligomer to at least two enzymes which function in a linked pathway.

The invention also encompasses a method of enhancing a reaction between at least two binding partners comprising coupling the binding partners to an HDAG oligomer. In a different embodiment, the method of enhancing a reaction between two binding partners comprises coupling one binding partner to an HDAG oligomer and

5 contacting the oligomer to a second binding partner.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts sequence alignment of 11 serotypes of hepatitis delta antigen (HDAG) between amino acids 12 to 60. Asterisks, *, indicate residues which make up the a and d positions in the heptad repeat in the predicted coiled-coil region. Bold pink and purple indicate residues involved in the hydrophobic interactions in the dimer

10 between the two termini. The "ds" indicate residues involved in the dimer-dimer interface. A region (pink), B (green), C (purple).

Figure 2 depicts the final atomic model superimposed upon a portion of the final 1.8 Å resolution $2F_o - F_c$ map. The map is contoured at 1.2σ and shows the residues

15 involved in the interaction between monomers at the A and C regions. Orientation is similar to that in Figure 4. Yellow indicates carbon, red indicates oxygen and blue indicates nitrogen. The figure was produced using BOBSCRIPT.

Figure 3A depicts $C\alpha$ trace of the peptide $\delta_{12-60}(Y)$. A region pink, B region green, and C region purple. The individual helix takes a sharp bend at proline 49

20 (Pro49). Figure 3B is a ribbon diagram of the view in Figure 3A rotated 90° along the horizontal axis. The sidechains have been added and the C region of the peptide (residues 50-60(Y)) has been removed for clarity. Sidechains are colored as follows: hydrophobic gray, polar yellow, acidic red and basic blue. Figure 3C is the amino acid sequence of the long helix formed from residues 12 to 48 displayed in the antiparallel

25 orientation of the peptide. The letters above the amino acid sequence represent the heptad repeat $(abcdefg)_n$ where the a and d residues tend to be hydrophobic. The residues involved in the heptad repeat at the a and d positions are shown in bold.

Figure 4 depicts the monomer-monomer interactions. The regions are colored as follows: A region pink, B region green, and C region purple. The white row of X's indicates a hydrogen bond between the sidechain of Glu45 (E45) and the indole nitrogen of Trp20 (W20). This figure was produced using RIBBONS, Carson, M. & Bugg, C.E., *J. Mol. Graphics*, 4:121-122 (1986).

Figure 5 depicts the interactions of dimers in the P2₁2₁2 unit cell. The unit cell is outlined in black and the directions of the A and B axes are shown. The two independent copies of the dimer in the asymmetric unit are colored orange and blue. The view is looking down the crystallographic 2-fold axis. This figure was produced using the program MOLSCRIPT (Kraulis, P.J. *J. Appl. Cryst.* 24:946-950 (1991)).

Figure 6 depicts the dimer-dimer interface. Figure 6A illustrates that the dimer-dimer interface is composed of a four-helix bundle made of the N and C termini of two dimers, one from across the crystallographic two-fold axis. One (unlabeled) dimer is colored yellow and the other (labeled) dimer is colored according to the scheme used in Figure 1. Figure 6B depicts the view in Figure 6A rotated 90° around the y axis. This figure was created with RIBBONS (Carson, M. & Bugg, C.E., *J. Mol. Graph.* 4:121-122 (1986)).

Figure 7A is a GRASP electrostatic potential surface of the octameric δ 12-60(Y) peptide contoured at = 10kT/e (positive potential blue) and = 10kT/e (negative potential in red) (K is Boltzman's constant and T is temp °K . The edges and the lining of the large 50Å hole are basic. Figure 7B illustrates the hole formed by the octamer.

Figure 8 graphically illustrates MALDI-TOF mass spectrometry analysis of recombinant small delta antigen (r-HDAg-S) (Figure 8A), and the glutaraldehyde cross-linked protein (Figure 8B).

Figure 9 depicts the sequence of a synthetic gene for optimized expression of HDAg-S in *E.coli*. The synthetic gene has been modified such that the codon usage which is unusual in the natural gene is assistant with the known preferences for codon usage in *E. coli*. The underlined sequences correspond to the eight primers used in the

first round of PCR. The primers used in the second round are indicated with a dotted underline. The amino acid sequence is shown above the DNA sequence by the one-letter amino acid code. The restriction sites used in cloning are shown in italics.

Figure 10 depicts the complete sequence of human HDV cDNA, and the
5 predicted amino-acid sequence of human HDV delta antigen.

Figure 11 depicts synthetic peptides from the multimer-forming domain of HDAg. (A) Structural organization of HDAg. The lightly stippled region is the multimer-forming domain (amino acid residues 12-60), the solid regions are the RNA-binding domains, and the heavily stippled region is the C-terminal extension of large
10 HDAg. Hydrophobic residues contributing to the heptad repeat are shown in boldface type, (B) Amino acid sequences of three HDAg peptides.

Figure 12 depicts use of the delta antigen as a scaffold. A construct containing eight appended protein domains on the octameric framework. Figure 12A depicts an oligomerization domain and spheres which represent potential effectors/ligands/nucleic acid binding domains etc. that have been fused to the C-terminus of the oligomerization domain of the delta antigen. Figure 12B depicts binding domain which is a multimer, specifically a tetramer. A similar fusion of up to eight effector/legands/nucleic acid binding domains could be made at the N-terminus, and constructs with fusion domains at up to eight N-termini and up to eight C-termini could also be made.
15

Figure 13 depicts plasmids comprising HDAg for expression in bacteria. "DAg" refers to a delta antigen sequence (with or without nuclear localization sequence); "MCS1" refers to a multiple cloning site for insertion of a heterologous gene at N-terminal end of delta antigen; "MCS2" refers to a multiple cloning site for insertion of a heterologous gene a C-terminal end of delta antigen; "Ori" refers to the origin of
20 replication for the bacteria; "drug" refers to a drug marker; "fl ori" refers to origin of replication of single-stranded DNA by a bacteriophage; "promoter" refers to bacterial promoter. Figure 13A depicts MCS1 3' to HDAg; Figure 13B depicts MCS1 5' to HDAg; and Figure 13C depicts MCS1 3' and MCS2 5' to HDAg.

Figure 14 depicts a plasmid comprising HDAG for expression in eukaryotic cells. "Dag refers to a delta antigen sequence (with or without nuclear localization sequence). "MCS1" refers to plasmids comprising replication of plasma in bacteria; "Ori" refers to origin of expression or replication of the plasmid in bacterial cells; "drug1" refers to a drug marker (e.g. ampicillin, kanamycin) for propagation of plasmid in *E. coli*; "drug2" refers to a drug marker or eukaryotic drug resistance (e.g. neomycin, zeocin, hybromycin), for propagation of plasmid in a eukaryotic cell; "fl ori" refers to origin of replication of single-stranded DNA by bacteriophage; "promoter" refers to eukaryotic promoter.

Figure 15 is a comparison of the wildtype nucleotide sequence of HDAG-S and the sequence of the synthetic HDAG gene for optimized expression in *E. coli*.

Figure 16 is the nucleotide sequence of the synthetic open reading frame (ORF) for the synthetic HDAG.

Figure 17 is a comparison of the protein amino acid sequence encoded by the wildtype ORF and the synthetic ORF, showing complete (100%) identity.

Figure 18 depicts the nucleotide sequences of the primers used for the two polymerase chain reactions (PCR) to create the synthetic gene. Primer1 - primer8 were used in the first round of PCR and primer9 - primer10 were used in the second round of PCR.

DETAILED DESCRIPTION OF THE INVENTION

As set forth above, the present invention relates to the discovery of the oligomeric structure of the hepatitis delta antigen (HDAG) which serves as a convenient high-valency framework for linking a variety of binding partners, including functional peptides and domains. The structure of the antigen includes a doughnut-shaped octamer comprising N-terminal antiparallel coiled-coil domains and stabilizing C-terminal domains. The invention includes HDAG proteins, including derivatives, mutants and fragments, and nucleic acid molecules encoding HDAG. It also includes an altered

HDAG gene for the capsid protein wherein the codons conform to use preferences for *E. coli*. Included in the derivatives are fusion molecules, e.g., fusion proteins, in which one or more binding moieties are attached to one or both termini of a monomer and coiled-coil oligomers (e.g., octamers) formed from the monomers. Coiled-coil

5 oligomers of the present invention can comprise one or more fusion molecules as described herein. The binding moieties can be, for example the same (homologous) or different (heterologous) binding partners. The invention also includes vectors and cassette expression systems which can be used to produce the fusion molecules. The vectors comprise HDAG and one or more binding moieties which are operably linked to

10 HDAG. The invention also relates to cells comprising HDAG nucleic acid, e.g. cells transformed with such vectors, and to methods of producing such cells. The invention also includes therapeutic and diagnostic methods involving HDAG.

HDAG PEPTIDES

HDAG, as defined herein, includes both the large and small delta antigens

15 (HDAG-S and HDAG-L). HDAG encompasses native ("wild type") proteins and also includes derivatives, mutations, and functional protein or polypeptide fragments of the native protein and/or proteins or polypeptides where one or more amino acids have been deleted, added or substituted.

HDAG can be isolated and/or purified, or it can be recombinant or prepared by

20 synthetic techniques described herein or known to those of skill in the art. HDAG proteins or fragments can be isolated from the cell of origin or produced synthetically or recombinantly. In a preferred embodiment, the protein is isolated to the substantial absence of conspecific proteins. A conspecific protein is a protein other than HDAG which can be obtained from the cell of origin for the protein or its nucleic acid. The

25 proteins (and nucleic acids) described herein can be preferably isolated, by known methods, to a purity of at least about 50% by weight, more preferably at least about 75%

and most preferably to substantial homogeneity. "Substantial homogeneity" refers to the substantial absence of conspecific proteins.

An HDAG peptide can, e.g., include all or a portion of the amino acids depicted Figures 1, 3C, 10, 11 or 17. An HDAG peptide can be encoded by an isolated and/or
 5 purified or recombinant nucleic acid molecule or a fusion gene (nucleic acid molecule) such as those described herein. In a preferred embodiment, an isolated and purified polypeptide has an amino acid sequence depicted in a row of Figure 1, amino acids 12-48 of a row of Figure 1, amino acids 12-60 of a row of Figure 1, a row of Figure 3C, Figure 9, amino acids 12-48 of Figure 9, amino acids 12-60 of Figure 9, Figure 10,
 10 Figure 11, Figure 17 or a fragment or derivative thereof, which forms a coiled-coil oligomer.

In another embodiment, an isolated and purified polypeptide has the amino acid sequence of amino acids 12-88 of HDAG, or a fragment or derivative thereof, which forms a coiled-coil oligomer and nuclear localization signal.

15 "Homology" is defined herein as sequence identity. Preferably, the protein or polypeptide shares at least about 50 % sequence identity or homology and more preferably at least about 75 % identify or at least about 90% identity with the corresponding sequences of the native protein, for example, with Figure 10. The phrase "substantially the same sequence" is intended to include sequences which bind the viral
 20 protein and possess a high percentage of (e.g., at least 90%, preferably at least about 95%) amino acid sequence identity with the native sequence. For example, a derivative, e.g., a mutant or variant can possess substantially the same amino acid sequence as the native protein.

The modifications to the amino acid sequence (substitutions) can be conserved
 25 or non-conserved, natural or unnatural amino acids. The residues that function to form or stabilize the coiled-coil domain or binding sites thereof can be substituted, e.g., conservatively, or they can be maintained. Amino acids of the native sequence for substitution, deletion or conservation can be identified, for example, by a sequence

alignment between proteins from different serotypes from related species or other related proteins. In one embodiment, the amino acids which are deleted, added or substituted are amino acids which are not "conserved" between serotypes or species, for example, the amino acids so identified in the sequence alignment exemplified in Figure

- 5 1. Conserved amino acids may also be substituted. In one embodiment they are substituted conservatively, for example, substituted by structurally similar amino acids. The phrase "conservative amino acids substitutions" is intended to mean substitutions of amino acids which possess similar side chains (e.g., hydrophobic, hydrophilic, basic acidic, aromatic, and aliphatic) as is known in the art. See, for example, Hermanson,
10 G.I. Bioconjugate Techniques, Academic Press, Inc. San Diego, CA (1996). Conservative substitutions include amino acid substitutions of one hydrophobic amino acid for another, for example within the following grouping: W, F, A, P, L, M, I, V. Acidic amino acids include E and D; basic amino acids include K, R, and H. Polar amino acids include S, T, N, Q and G and amide residues include Q and N. An example
15 of a suitable derivative or mutant of the HDAG protein is a protein possessing a consensus sequence of the originating species.

In one embodiment, the derivative does not contain substitutes of the residues of Arg13, Leu17, Trp20, Arg24, Trp50 or Leu51. In another embodiment, it contains only conservative substitutions in this region. Hydrophobic residues, for example Ile16,

- 20 Leu17, Trp20, Trp50 and Leu51 can be maintained, or they can be replaced with other hydrophobic amino acids, for example, those from the group consisting of Trp, Phe, Ala, Pro, Leu, Met, Ile and Val. In another example, the residues Glu31, Lys38, Trp20 and Glu45 are not substituted or are substituted conservatively. In addition, Arg13 and Arg24 can be maintained (not substituted) or substituted conservatively. In another
25 embodiment, the residues of Figure 1 which are involved in hydrophobic interactions are substituted with other hydrophobic residues. In Figure 3A, hydrophobic residues can be substituted for other hydrophobic residues, polar residues can be substituted for other polar residues, acidic residues can be substituted for other acidic residues, and/or

basic residues for other basic residues. In one example, the residues labeled in Figure 3A can be maintained (not substituted) or can be replaced with amino acids with similar characteristics. The amino acids at the 'a' and 'd' positions of the heptad repeat (for example, those indicated with an asterisk in Figure 1 or those listed in bold in Figure 3C), can be conserved (maintained), or they can be substituted conservatively, e.g., replaced with hydrophobic amino acids. The residues involved with the dimer-dimer interface (e.g., residues marked with a 'd' in Figure 1 or residues labeled in Figure 6) can be maintained. The residues indicated in Figure 4 can be maintained. The derivatives, e.g. mutant and wild-type peptides, can crystallize isomorphously. In one preferred embodiment, at least one serine residue, e.g., Ser22, is replaced with a cysteine. In another embodiment, Trp20 is replaced with Ala20.

A variety of substitutions based on amino acid characteristics can be made. For example, the polar amino acid residues can be substituted and the hydrophobic amino acids can be maintained. In addition the nonhydrophobic residues can be substituted and the hydrophobic residues can be maintained. In one embodiment, a derivative can comprise substitutions of any or all of the amino acid residues in the following positions: 14, 15, 18, 19, 22, 24, 25, 26, 28, 29, 31, 32, 33, 35, 36, 38, 39, 40, 42, 43, 45, 46 and 47. The nonhydrophobic residues can be substituted such that acidic amino acids are alternated with basic amino acids. The hydrophilic residues in the C terminal region can be substituted to optimize stability of the helix, for example by presenting one or more amino acids which form disulfide bonds, strong ionic bonds or cross-linked moieties, with the corresponding amino acid of another subunit. In one embodiment, residues 53, 55 and 60 are substituted.

Especially preferred are derivatives, e.g., coiled-coil subunits, which improve (e.g., optimize) stability of the coiled-coil structure or which improve cross-linkage involving the structure, or which improve the ability of the structure to be immobilized on a solid substrate.

The term derivative is also intended to include proteins which have been labeled, such as with a radioactive or colorimetric label. Such derivatives are more readily

detected in an assay. In one embodiment, a peptide is synthesized that corresponds to residues 12-60 of HDAG, and includes a C-terminal tyrosine, enabling the peptide to be labeled, e.g., with I²⁵, for use in a radioimmunoassay. In one embodiment, the peptide is δ 12-60(Y). Yet other derivatives are proteins which consist essentially of the amino acid sequence of a given protein (e.g., possess the relevant sequence and, optionally, other amino acids residing at the termini which do not significantly alter or detract from the properties of the protein).

A "functional" fragment, derivative, mutant, or allelic variant is of sufficient length and/or structure as to possess one or more biological activities of the protein.

- 10 One example of such a biological activity of the protein is formation of a coiled-coil oligomer, e.g., an octamer, for example, an octamer doughnut-shaped structure. In one embodiment, the protein derivative is conserved within the coiled-coil regions but is lacking in or mutated within one or more other regions (e.g., sequences not within the coiled-coil. Examples of suitable fragments include peptides lacking fragments which
- 15 encode or stabilize the coiled coil, for example, amino acids 12-48, or the peptides depicted in Figure 11. One example includes fragments which lack all or part of the region C-terminal to the proline bend (e.g. C-Terminal to Pro49). Another fragment includes the coiled coil and nuclear localization signal (e.g., amino acids 12-88); or solely the nuclear localization signal (amino acids 68-88). Xia *et al.*, *J. Virol.* 66:914-
- 20 21 (1992). Yet another example includes HDAG which encodes the coiled-coil region but is lacking all or a portion of the nuclear localization signal. In one embodiment, all or a portion of one or both termini of a monomer is absent or mutated. For example, the C region of the peptide (e.g., residues 50-60) can be mutated or all or a portion can be eliminated. Yet another example of derivatives includes peptides which possess amino
- 25 acid modifications or additions which are characterized by a functional group which can react with a compound substituted by a "binding moiety", such as those described above or with a cross-linking agent.

Yet other biologically activities are the ability of HDAG-S to function as a *trans* activator of replication and the ability of HDAG-L to act as an inhibitor of replication.

In yet another embodiment, the biological activity of the protein is antigenic or immunogenic activity.

Fragments of the protein can possess at least about 10 amino acids from the 12-48 amino acid region, preferably at least about 20 amino acids. In other embodiments, the fragment possesses essentially all of the amino acids of the full-length protein (e.g., at least about 85%, or at least about 95%).

HDAG includes monomers and oligomers, e.g., dimers and octamers, comprising the monomers as subunits. The invention encompasses HDAG coiled-coil oligomers, e.g. octamers.

10 FUSION MOLECULES

Fusion molecules are intended to be included within the definition of HDAG derivatives and can be made by linking one or more binding moieties, e.g., chemicals or peptides, to the HDAG protein or fragment, for example, through a covalent bond or preferably a peptide bond or cysteine group. As such, derivatives, such as fusion proteins, can comprise the amino acid sequence of the HDAG protein or fragment and a binding moiety such as a given protein, e.g., a native protein.

A "binding moiety," as the term is defined herein, includes a chemical entity which is bound to HDAG. The binding can be via a covalent bond (e.g. through a cysteine group), ionic bonding, hydrogen bonding or other mechanism. The binding moiety and the HDAG can be expressed as a single unit. The binding moiety can be a peptide (including post-translationally modified proteins, such as amidated, demethylated, glycosylated or phosphorylated proteins), sugar, lipid, steroid, nucleic acid, small molecule, anion or cation, drug, chemical or combination thereof which binds the specified binding partner (e.g., a target molecule). Preferably, the binding will possess a high affinity. Examples of high affinity can have a dissociation constant of 10^{-5} M (preferably 10^{-8} M) or lower. Examples of binding moieties include an antigen, an antibody, a ligand, a receptor, an enzyme, a (ligand) interaction peptide, a chemical, an effector, an oligonucleotide, a signal amplification peptide, an enhancer recognition

protein, a promoter binding protein, a label, a growth factor, a cytokine, a nuclease, a small organic molecule, a test substance, a cytotoxic agent, a substrate, a solid substrate, a drug or a fragment thereof.

Where there are two or more binding moieties, the binding moieties can be the same (homologous) or different (heterologous). The binding moieties can be binding partners. Examples of binding partners include, but are not limited to, antigen-antibody and ligand-receptor. First and second binding moieties can also include the following pairs: enzyme1-enzyme2, (ligand) interaction peptide-effector peptide (or chemical), oligonucleotide-nuclease, interaction agent (e.g. peptide signal amplification agent, (e.g. peptide), enhancer recognition agent (e.g. protein)-promoter-binding agent (e.g. protein), enhancer recognition agent-promoter binding agent, ligand-label, test substance-label, targeting agent - effector agent, drug (or hormone) -label, or any other combination.

In one embodiment, a first binding moiety binds, a target molecule on a target cell (e.g. a surface protein) and the binding partner is the surface protein or target cell. The "target cell" is defined as the cell which is intended to be contacted by the fusion cell. Typically, the target cell is of animal origin and can be a stem cell or somatic cell. Suitable animal cells for use on the claimed invention can be of, for example, mammalian and avian origin. Examples of mammalian cells include human, bovine, ovine, porcine, murine, rabbit cells. The cell may be an embryonic cell, bone marrow stem cell or other progenitor cell. Where the cell is a somatic cell, the cell can be, for example, an epithelial cell, fibroblast, smooth muscle cell, blood cell (including a hematopoietic cell, red blood cell, T-cell, B-cell, etc.), tumor cell, cardiac muscle cell, macrophage, dendritic cell, neuronal cell (e.g., a glial cell or astrocyte), or pathogen-infected cell (e.g., those infected by bacteria, viruses, virusoids, parasites, or prions).

Typically, cells isolated from a specific tissue (such as epithelium, fibroblast or hematopoietic cells) are categorized as a "cell-type." The cells can be obtained commercially or from a depository or obtained directly from an animal, such as by

biopsy. Alternatively, the cell need not be isolated at all from the animal where, for example, it is desirable to deliver the vector to the animal in gene therapy.

Cells can typically be characterized by markers expressed at their surface that are termed "surface markers". These surface markers include surface proteins or target molecules, such as cellular receptors, adhesion molecules, transporter proteins, components of the extracellular matrix and the like. These markers, proteins and molecules also include specific carbohydrates and/or lipid moieties, for example, conjugated to proteins. In one embodiment, a binding moiety on a fusion molecule can bind to one or more surface proteins on the target cell. Surface proteins can be tissue- or cell-type specific (e.g. as in surface markers) or can be found on the surface of many cells. Typically, the surface marker, protein or molecule is a transmembrane protein with one or more domains which extend to the exterior of the cell (e.g. the extracellular domain). Where cell-type specific delivery is desired (as in *in vivo* delivery of a drug), the surface protein selected for the invention is preferably specific to the tissue. By "specific" to the tissue, it is meant that the protein be present on the targeted cell-type but not present (or present at a significantly lower concentration) on a substantial number of other cell-types. While it can be desirable, and even preferred, to select a surface protein which is unique to the target cell, it is not required for the claimed invention. It is to be appreciated, however, that specific delivery may not be required where the cell or cells are contacted with the viral vector in pure or substantially pure form, such as can be the case in an *in vitro* gene transfer. As such, the surface protein or targeted protein for the first binding moiety may be present on many different cell-types, specific or even unique to the targeted cell-type.

As set forth above, the surface protein can be a cellular receptor or other protein, preferably a cellular receptor. Examples of cellular receptors include receptors for cytokines, growth factors, and include, in particular epidermal growth factor receptors, platelet derived growth factor receptors, interferon receptors, insulin receptors, proteins with seven transmembrane domains including chemokine receptors and frizzled related proteins (Wnt receptors), immunoglobulin-related proteins including MHC proteins,

CD4, CD8, ICAM-1, etc., tumor necrosis factor-related proteins including the type I and type II TNF receptors, Fas, DR3, DR4, CAR1, etc., low density lipoprotein receptor, integrins, and, in some instances, the Fc receptor.

Other examples of surface proteins which can be used in the present invention include cell-bound tumor antigens. Many of these surface proteins are commercially available and/or have been characterized in the art, including the amino acid and nucleic acid sequences, which can be obtained from, for example, GENBANK, as well as the specific binding characteristics and domains. Cytokine and chemokine receptors are reviewed for example, in Miyama, *et al. Ann. Rev. Immunol.*, 10:295-331 (1992),
10 Murphy, *Ann. Rev. Immunol.* 12:593-633 (1994) and Miller *et al. Critical Reviews in Immunol.* 12:17-46 (1992).

The binding moiety can be selected or derived from native ligands or binding partners to the surface protein of the target cell. In the case of a cellular receptor, for example, for a cytokine or growth factor, the binding moiety can be a polypeptide comprising at least the receptor-binding portion of the native ligand. A "native ligand" or "native binding partner" is defined herein as the molecule naturally produced by, for example, the animal or species which binds to the surface protein in nature. Preferably, the binding moiety is a polypeptide or protein. As such, the native ligand of a cytokine receptor can be the native cytokine. In another embodiment, the binding moiety can
15 comprise a binding fragment of an antibody, such as the variable region or a single chain antibody.

Where a binding moiety comprises a binding fragment of an antibody, many antibodies to surface proteins are known or are commercially available, as are the amino acid sequences which are responsible for binding. Alternatively, novel antibodies can
25 be prepared by methods known in the art, such as by Harlow and Lane, "Antibodies, A Laboratory Manual," Cold Spring Harbor Laboratory (1988). The binding fragment can comprise an antibody fragment, for example, the constant region or, the variable region (e.g., Fc fragment or FAb' fragment).

A binding moiety can be a polypeptide ligand to a cellular receptor. Examples of preferred ligands are growth factors, epidermal growth factor, interleukins, GM-CSF, G-CSF, M-CSF, EPO, TNF, interferons, and chemokines. In one embodiment, the receptor is a transferrin receptor.

- 5 The binding moiety can have an amino acid sequence which is the same or substantially the same as an amino acid sequence of at least the receptor-binding portion of a native ligand for the cellular receptor. Similar to cellular receptors, many of the corresponding ligands have been identified, sequenced and characterized, including the portions thereof which bind to the receptor. The binding moiety can, therefore, include
- 10 the same or substantially the same sequence of the entire native ligand. Alternatively, binding moiety comprises the receptor binding portion of the native ligand, eliminating, in some cases, the effector function of the ligand.

- In another embodiment, the binding moiety is selected or derived from native ligands or binding partners to a cellular surface molecule of a target cell. A "cellular
- 15 surface molecule" as defined herein can be a peptide (including post-translationally modified proteins, such as amidated, demethylated, methylated, prenylated, palmitoylated, glycosylated, myristylated, acetylated or phosphorylated proteins), sugar, lipid, steroid, anion or cation, or a combination thereof which binds the first binding moiety. Preferably, the binding of the cellular surface molecule to the binding moiety
- 20 of the bifunctional molecule will be of high affinity. Examples of high affinity have a dissociation constant of 10^{-5}M (preferably 10^{-8}M) or better.

- The cellular surface molecule need not be "specific" for the target cell. However, the cellular surface molecule is specific for a desired viral vector. For example, specific delivery of Influenza A viral vectors can employ sialic acid cellular
- 25 surface molecules for entry into a target cell whereas targeting of VSV viral vectors can employ a phospholipid as the surface molecule. As such, the cellular surface molecule for the first binding moiety can be present on many different cell-types, specific or even unique to the target cell.

In other embodiments, the effector function can be desirable, thereby stimulating or modulating the cellular activity of the target cell which can enhance therapy. An example of where such a therapy can be desirable is in the delivery of a negative selection marker or suicide protein to a tumor where the target cell is a lymphokine and the ligand is a cytokine. Where the lymphokine is stimulated, the cell, can also possess therapeutic value in the recruitment of an endogenous immune response against the tumor, thereby increasing the therapeutic benefit of the therapy.

The phrase "substantially the same sequence" is intended to include sequences which bind the surface protein and possess a high percentage of (e.g., at least about 90%, preferably at least about 95%) sequence identity with the native sequence. The modifications to the sequence can be conserved or non-conserved, natural and unnatural, amino acids and are preferably outside of the binding domain. Amino acids of the native sequence for substitution, deletion, or conservation can be identified, for example, by a sequence alignment between proteins from related species or other related proteins.

In addition to the first binding moiety, there can be a second binding moiety which is a chemical entity which binds to HDAG. The binding can be via a covalent bond, ionic bonding, hydrogen bonding or other mechanism. The second binding moiety can be the same or different from the first. For example, it can be a peptide, sugar, lipid, steroid, nucleic acid, small molecule, anion or cation, or combination thereof which binds the HDAG. In one embodiment, the second binding moiety of the fusion protein is also a polypeptide. One embodiment of the second binding moiety comprises an antigen-binding fragment of an antibody which recognizes and binds to an antigen.

Ligand receptors which are cellular receptors can be transmembrane proteins comprising intracellular, transmembrane (characterized by highly hydrophobic regions in the sequence) and extracellular domains. In one embodiment, the second binding moiety can comprise the native extracellular domain of a receptor molecule.

Fusion proteins can be made conveniently through known methods, e.g. recombinantly. The binding moieties can be directly bonded to HDAG or can be bonded to HDAG through a linking moiety. Where one or both of the moieties are polypeptides, a peptide bond or peptide linker may be preferred, thereby obtaining a "fusion protein".

- 5 The "fusion protein" of the HDAG and one or more moieties can be expressed by a single nucleic acid construct in series. One or more moieties and HDAG alternatively be linked directly or indirectly other than via a peptide bond or peptide linker, thereby obtaining a "conjugate".

- Where the moieties and HDAG are directly bonded to each other, the bond can
10 be covalent, as in a peptide bond, ionic bond or hydrogen bond. Where the bond is a peptide bond, a binding moiety can be bonded to the N terminus of HDAG via the C terminus, or vice versa or both. It is acknowledged that one fusion protein may possess greater activity than a second fusion protein due to conformational or steric considerations. The binding moieties can be, for example, monomers, dimers and
15 tetramers.

- Where one or more of the binding moieties are not polypeptides, they can be joined via chemical reaction through functional groups present on each moiety which, under the appropriate conditions, will react with each other. For example, acid groups (or activated derivatives thereof) can be reacted with amines, alcohols or thiols to form
20 amide or ester bonds, as is known in the art.

- Alternatively and advantageously, a linking moiety is employed to link the binding moieties, e.g. binding partners, to HDAG. The linker can preferably be a flexible linker and sufficient in length to separate the moieties in space, thereby not restricting the ability of the fusion molecule to bind independently and maintain the
25 proper conformation. Again, where both moieties are polypeptides, the linker moiety will generally be a peptide, polypeptide, or a "pseudopeptide". A "pseudopeptide" is a bifunctional linker which contains at least one non-amino acid and reacts to form a peptide bond, or other bond, with the terminal amine or carboxyl group of the moiety. For example, a peptide characterized by substitution of the terminal amine for a

carboxyl group can function to react with the amine terminus of each moiety. Such as linker is considered to be a "pseudopeptide." Similarly, a peptide characterized by substitution of the terminal carboxyl for an amine group can function to react with the carboxyl terminus of each moiety.

- 5 Generally, however, the linker will be a peptide linker which will link the amine terminus of a moiety to the carboxyl terminus of HDAG or vice versa. One advantage to such a molecule is the ability to express the fusion protein in a recombinant host cell with a single nucleic acid construct.

- 10 Peptide linkers can be obtained from immunoglobulin hinge regions, such as a proline-rich region. Also, linkers can be characterized by little steric hindrance, thereby permitting maximal independent movement of the two moieties, such as with a polyglycine linker. Alternatively, the linker selected to be reactive to or inert to cellular proteases can be desirable. In another embodiment, the linker can be selected to avoid or minimize an immune response against the fusion molecule. The length of the linker
15 also is not particularly critical. Typically, the length of the linker can be between about 2 and about 20 amino acids. As can be seen, the selection of the particular linking group is not critical to the invention.

- 20 In yet another embodiment, the linker can be a bifunctional compound which will react with other functional groups on the binding moieties or HDAG, such as in the reaction of acids and amines or alcohols (as present in peptides, carbohydrates and lipids, for example) in the formation of amides or esters.

- A preferred combination of the above first and second binding moieties includes one binding partner, e.g. a polypeptide ligand to a cell-type specific cellular receptor linked, via a peptide linker, through a terminus of the ligand to the terminus of HDAG.
25 A second binding partner, e.g. a extracellular domain of a cellular receptor or a mutant thereof, can be linked to the same or different HDAG subunit in the same manner.

For example, the C terminus of a binding polypeptide is linked to the N terminus of HDAG via the polypeptide linker or the N terminus of the first binding polypeptide is linked to the C terminus of the HDAG via the polypeptide linker.

In another aspect of the invention, peptidomimetics (molecules which are not polypeptides, but which mimic aspects of their structures to bind to the same site) that are based upon the above-described polypeptides, can also be used. For example, polysaccharides can be prepared that have the same functional groups as the polypeptides of the invention, and which interact with binding partners in a similar manner. Peptidomimetics can be designed, for example, by establishing the three =
10 dimensional structure of the polypeptide in the environment in which it is bound or will bind to the binding partner. The peptidomimetic can comprise at least two components, a binding entity or entities and a backbone or supporting structure entity.

The binding entities of the peptidomimetic are the chemical atoms or groups which will react or complex (as in the formation of a hydrogen or covalent bond) with a
15 binding partner. In general, the binding entities in a peptidomimetic are the same as the polypeptide moieties. Alternatively, the binding entities can be an atom or chemical group which will react with the binding partner in the same or similar manner as the polypeptide. Examples of binding entities suitable for use in designing a peptidomimetic for a basic amino acid in a polypeptide are nitrogen containing groups,
20 such as amines, ammoniums, guanidines and amides or phosphoniums. Examples of binding entities suitable for use in designing a peptidomimetic for an acidic amino acid in a polypeptide can be, for example, carboxyl, lower alkyl carboxylic acid ester, sulfonic acid, a lower alkyl sulfonic acid ester or a phosphorous acid or ester thereof.

The supporting structure is the chemical entity that, when bound to the binding
25 moiety or moieties, provides the three dimensional configuration of the peptidomimetic. The supporting structure can be organic or inorganic. Examples of organic supporting structures include polysaccharides and polymers (such as, polyvinyl alcohol or polylactide). It is preferred that the supporting structure possess substantially the same

size and dimensions as the polypeptide backbone or supporting structure. This can be determined by calculating or measuring the size of the atoms and bonds of the polypeptide and peptidomimetic. For example, the nitrogen of the peptide bond can be substituted with oxygen or sulfur, thereby forming a polyester backbone. Likewise, the carbonyl of the peptide bond can be substituted with a sulfonyl group or sulfonyl group, thereby forming a polyamide. Reverse amides of the peptide can be made (e.g., substituting one or more -CONH- groups for a -NHCO- group). In addition, the peptide backbone can be substituted with a polysilane backbone.

These peptidomimetic compounds can be manufactured by art-known and art-recognized methods. For example, a polyester corresponding to a given peptide can be prepared by the substituting a hydroxyl group for each corresponding amine group on the amino acids, thereby preparing a hydroxyacid and sequentially esterifying the hydroxyacids, optionally blocking the basic side chains and acids to minimize side reactions. Determining an appropriate chemical synthesis route can generally be readily identified upon determining the chemical structure using no more than routine skill.

The fusion molecules can be manufactured according to methods generally known in the art. For example, where one or both of the binding moieties is a nonpeptide, the fusion molecule can be manufactured employing known organic synthesis methods useful for reacting a functional or reactive group on the moiety with a functional or reactive group on the other moiety or, preferably, a linker. In carrying out the synthesis, derivation or inactivation of the functional group(s) required for binding to the moiety's binding partner should be avoided. Appropriate syntheses are highly dependent upon the chemical nature of the binding moiety and, generally, can be selected from an organic chemistry text, such as March *et al. Advanced Organic Chemistry*, 3rd Edition (1985) John E. Wiley & Sons, Inc., New York, NY, or other known methods.

Where the binding moieties are polypeptides, the fusion molecule can be a conjugate or a fusion protein and manufactured according to known methods. Where a

fusion protein is desired, the molecule can be manufactured according to known methods of recombinant DNA technology. For example, the fusion protein can be expressed by a nucleic acid molecule comprising sequences which code for both moieties, such as by a fusion gene (nucleic acid molecule). Thus, the invention further
5 relates to nucleic acid molecules, including fusion genes, which encode HDAG fragments, mutants and derivatives.

NUCLEIC ACID MOLECULES

Recombinant or isolated nucleic acid molecules of the invention, in one embodiment, encode an HDAG protein (including the e.g., native proteins, fragments,
10 derivatives, mutants and allelic variants) as defined herein. A nucleic acid molecule of the present invention can be double-stranded or single-stranded and can be a DNA molecule, such as cDNA or genomic DNA, or an RNA molecule. The nucleic acid molecule can be placed in a construct, which can be inserted into a vector. As such, the nucleic acid molecule can include one or more exons, with or without, as appropriate,
15 introns. In one embodiment, the nucleic acid molecule contains a single open reading frame which encodes HDAG and one or more binding moieties and, optionally, a signal sequence and/or a polypeptide linker, when present. By way of example in a multi-exon construct, the nucleic acid molecule contains a first exon which begins with an ATG, encodes a binding moiety, and optionally the polypeptide linker, and ends with a splice
20 donor site. The construct would also contain an HDAG-coding nucleic acid sequence and would further would contain an intron followed by a second exon which begins with a splice acceptor site and, optionally, a polypeptide linker, coding sequences for a second binding moiety and ending with a stop codon. Alternative combinations of these elements would be apparent to the person of skill in the art.

25 As such, the nucleic acid molecule can include sequences which encode HDAG, and one or more moieties, as well as one or more of the following optional sequences, in a functional relationship: regulatory sequences (as will be discussed in more detail

below) a start codon, a signal or leader sequence, splice donor sites, splice acceptor sites, introns, a stop codon, transcription termination sequences, 5' and 3' untranslated regions, polyadenylation sequences, negative and/or positive selective markers, and replication sequences.

5 The coding regions of the nucleic acid molecule code for HDAG and the binding moiety or moieties and any polypeptide linkers present. Where the binding moiety is a native ligand or cellular surface protein (e.g. a cellular receptor), or a binding fragment thereof, the nucleic acid molecule coding regions can correspond to the native sequences which encode a binding moiety. Because many amino acids are encoded by a plurality of codons, the coding sequence can be mutated to result in the same amino acid sequence. This may be advantageous where a codon is preferred by the selected host cell. In one embodiment, the HDAG gene can be altered such that the codons conform to the known codon use preferences for *E. coli*. See Figure 9 and Figures 15-17. The gene can be inserted into a convenient expression vector which allows production of several forms of the capsid protein including residues 1-84 (terminated in the middle domain), the short isoform and the long isoform. Dingle *et al.*, *J. Virol.* (1998). All three forms express well. Preferably, the nucleic acid molecule comprises the or corresponding coding nucleotide sequence of Figure 9, 10, 15-16, or substantially the same sequences thereof, or the complement thereof. In another embodiment, the nucleic acid molecule does not possess the nucleotide sequence of GenBank Accession #M28267. The nucleic acid molecule can be, for example, isolated and/or purified or recombinant.

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 In a preferred embodiment, the nucleic acid molecule comprises the nucleotide sequence depicted in Figure 9, nucleotides 37-150 of Figure 9, nucleotides 37-186 of Figure 9, Figure 10, nucleotides 1421-1566 of Figure 10 or nucleotides 1457-1566 of Figure 10, Figure 15, Figure 16; or a fragment or mutation thereof, which encodes a coiled-coil oligomer. In another preferred embodiment, the nucleic acid molecule comprises a nucleotide sequence encoding a polypeptide comprising an amino acid

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sequence depicted in a row of Figure 1, amino acids 12-48 of a row of Figure 1, Figure 3C, Figure 9, amino acids 12-48 of a row of Figure 9, Figure 10, amino acids 12-88 of Figure 10, Figure 9, or δ 12-60(Y). Also encompassed in the invention are complementary strands of these sequences, DNA sequences that hybridize to these sequences and RNA sequences transcribed from these sequences. Also included are fragments or mutations thereof, which encode a coiled-coil oligomer.

In one embodiment, the nucleic acid molecule encodes a polypeptide of a fusion molecule described herein.

Also included are fusion nucleic acid molecules (e.g. fusion genes) comprising an HDAG nucleic acid molecule operably linked to a heterologous nucleic acid molecule ("heterologous gene"), which encodes a peptide which is not HDAG and not derived therefrom (i.e., "heterologous protein"). Where the binding moiety is a mutation or variant of a native sequence, as provided above, generally, the nucleic acid sequence can be mutated correspondingly. It may also be preferred for ease of manufacture of the nucleic acid sequence to maintain as much of the native sequence as possible. In one embodiment, the nucleic acid molecule shares at least about 50% sequence identity with the corresponding native sequence such as the coding region, for example, the coiled-coil region, e.g., amino acids 12-48 or amino acids 12-60. In one embodiment, the sequence identity is at least about 65%, more preferably, 75%. In a more preferred embodiment, the percent sequence identity is at least about 90%, and still more preferably, at least about 95%.

Recombinant nucleic acid molecules meeting these criteria comprise nucleic acids having sequences identical to sequences of naturally occurring genes, including polymorphic or allelic variants, and portions (fragments) thereof, or variants of the naturally occurring genes. Such variants include mutants differing by the addition, deletion or substitution of one or more residues, modified nucleic acids in which one or more residues are modified (e.g., DNA or RNA analogs), and mutants comprising one or more modified residues.

Many nucleic acid molecules coding for suitable binding moieties are known in the art and can be obtained from, for example, GENBANK. Alternatively, other sequences can be employed, such as homologs of known genes.

Such homologous nucleic acids, including DNA or RNA, can be detected and/or
 5 isolated by hybridization (e.g., under high stringency conditions or moderate stringency conditions). "Stringency conditions" for hybridization is a term of art which refers to the conditions of temperature and buffer concentration which permit hybridization of a particular nucleic acid to a second nucleic acid in which the first nucleic acid may be perfectly complementary to the second, or the first and second may share some degree
 10 of complementarity which is less than perfect. For example, certain high stringency conditions can be used which distinguish perfectly complementary nucleic acids from those of less complementarity. "High stringency conditions" and "moderate stringency conditions" for nucleic acid hybridizations are explained on pages 2.10.1-2.10.16 (see particularly 2.10.8-11) and pages 6.3.1-6 in *Current Protocols in Molecular Biology*
 15 (Ausubel, F.M. *et al.*, eds., Vol. 1, containing supplements up through Supplement 29, 1995), the teachings of which are hereby incorporated by reference. The exact conditions which determine the stringency of hybridization depend not only on ionic strength, temperature and the concentration of destabilizing agents such as formamide, but also on factors such as the length of the nucleic acid sequence, base composition,
 20 percent mismatch between hybridizing sequences and the frequency of occurrence of subsets of that sequence within other non-identical sequences. Thus, high or moderate stringency conditions can be determined empirically.

By varying hybridization conditions from a level of stringency at which no hybridization occurs to a level at which hybridization is first observed, conditions which
 25 will allow a given sequence to hybridize (e.g. selectively) with the most similar sequences in the sample can be determined.

Exemplary conditions are described in Krause, M.H. and S.A. Aaronson, *Methods in Enzymology*, 200:546-556 (1991). Also, see especially page 2.10.11 in

Current Protocols in Molecular Biology (*supra*), which describes how to determine washing conditions for moderate or low stringency conditions. Washing is the step in which conditions are usually set so as to determine a minimum level of complementarity of the hybrids. Generally, starting from the lowest temperature at which only homologous hybridization occurs, each °C by which the final wash temperature is reduced (holding SSC concentration constant) allows an increase by 1% in the maximum extent of mismatching among the sequences that hybridize. Generally, doubling the concentration of SSC results in an increase in T_m of ~17°C. Using these guidelines, the washing temperature can be determined empirically for high, moderate or low stringency, depending on the level of mismatch sought. The following table provides an example of each condition of stringency.

Stringency	% Allowed mismatch	°C Temperature	% Formamide
High	6.6	52	50
Medium	13	45	50
Low	27	45	22

"Selective isolation", or "selective hybridization", is defined herein as embracing the isolation of a sufficiently few number of molecules (preferably one) as to readily permit the identification of the nucleic acid of interest.

The nucleic acid molecule also preferably comprises regulatory sequences.

Regulatory sequences include *cis*-acting elements that control transcription and regulation such as, promoter sequences, enhancers, ribosomal binding sites, and transcription binding sites. Selection of the promoter will generally depend upon the desired route for expressing the protein. For example, where the molecule will be introduced (e.g. transformed) into a cell by a viral vector, e.g. a plasmid, preferred

promoter sequences include viral, such as retroviral or adenoviral, promoters. Examples of suitable promoters include the cytomegalovirus immediate-early promoter, the retroviral LTR, SV40, and TK promoter. Where the molecule is to be expressed in a recombinant eukaryotic or prokaryotic cell, the selected promoter is recognized by the host cell. In one embodiment the construct is a cassette expression system. A suitable promoter which can be used can include the native promoter for the binding moiety which appears first in the construct.

The elements which comprise the nucleic acid molecule can be isolated from nature, modified from native sequences or manufactured *de novo*, as described, for example, in the above-referenced texts. The elements can then be isolated and fused together by methods known in the art, such as exploiting and manufacturing compatible cloning or restriction sites.

VECTORS AND HOST CELLS

The nucleic acid molecules can be inserted into a construct, e.g. a vector, such as a plasmid or cassette expression system, which can, optionally, replicate and/or integrate into a recombinant host cell, by known methods.

The vectors of the present invention comprise a nucleic acid molecule which encodes HDAG (e.g. an HDAG monomer). The monomer can be a subunit of an HDAG coiled-coil oligomer, e.g. an octamer. The oligomer can comprise an HDAG polypeptide as described herein. The nucleic acid molecule thus includes any of the nucleic acid molecules described herein, for example, a native (wild type) nucleic acid, or a fragment, mutant or derivative. Especially preferred are nucleic acids encoding full-length HDAG (e.g. HDAG-S or HDAG-L) or a fragment or derivative thereof, (e.g. a functional fragment) capable of forming a coiled-coil octamer (e.g. an N-terminal coiled-coil octamer). Preferred vectors comprise a nucleic acid molecule comprising nucleotide sequence depicted in Figure 9, nucleotides 37-150 of Figure 9, nucleotides 37-186 of Figure 9, Figure 10, nucleotides 1421-1566 of Figure 10 or nucleotides 1457-

1566, Figure 10, Figure 15 and Figure 16. Preferred vectors also comprise a nucleic acid comprising a nucleotide sequence encoding a polypeptide comprising an amino acid sequence depicted in a row of Figure 1, amino acids 12-48 of a row of Figure 1, the top row of Figure 3c, Figure 9, amino acids 12-48 of a row of Figure 9, Figure 10, amino acids 12-88 of Figure 10, Figure 11, Figure 17 or δ 12-60(Y). Other preferred vectors comprise nucleic acids comprising sequences which are the complementary strands of the above, DNA sequences which hybridize to these sequences, RNA sequences transcribed from these sequences, and fragments and mutations thereof, which encode a coiled-coil oligomer, e.g. an octamer. Vectors can also comprise fusion molecules comprising HDAG and at least one binding moiety, as described herein.

In a preferred embodiment, the vector additionally comprises at least one multiple cloning site. A multiple cloning site comprises a cleavage sites for commonly used restriction sites to facilitate incorporation of foreign (non-HDAG) gene, e.g. a cassette. A multiple cloning site can be located 3' or 5' to the nucleic acid molecule encoding HDAG. There can be multiple cloning sites, for example, there can be a multiple, cloning site 3' of the HDAG nucleic acid molecule and another multiple cloning site 5' to the HDAG nucleic acid molecule. The multiple cloning site can be located in a flanking region.

The vector can further comprise nucleic acid encoding a nuclear localization signal, e.g. an HDAG nuclear localization signal, for example, amino acids 68-88 of HDAG, as shown in Figure 9. The vector can also comprise an HDAG nucleic acid molecule comprising a sequence encoding a coiled coil and a nuclear localization signal.

The vectors of the invention can be used for the expression of a fusion molecule as described herein. A heterologous (non-HDAG) nucleic acid molecule, e.g., a gene, encoding a binding moiety of a fusion molecule can be inserted into a vector comprising HDAG, e.g., into a multiple cloning site. A vector can comprise one heterologous gene or more than one heterologous gene. The genes can be the same or different. A first

heterologous gene can encode a first binding moiety and a second heterologous gene can encode a second binding moiety. The first and the second binding moieties can be binding partners as described herein (for example, single chain antibody and antigen, ligand and receptor, components of a linked pathway, etc). The heterologous gene or
5 genes and the nucleic acid encoding HDAG can be operably linked, e.g., in the same open reading frame. Where HDAG nucleic acid and a heterologous gene encoding a binding moiety are operably linked, they are expressed as a single protein unit, i.e., a fusion molecule.

In one embodiment, a vector comprises an HDAG nucleic acid molecule (e.g. a
10 nucleic acid cassette) encoding a monomer capable of being a unit of a coiled-coil octamerization scaffold and a heterologous gene encoding a binding moiety, wherein the expressed binding moiety is bound to one terminal of the monomer, e.g. the N terminus or the C terminus. Where there are two expressed heterologous genes, each end of the monomer can be bound to an expressed binding moiety.

15 Vectors can additionally comprise a nucleic acid molecule encoding a nuclear localization signal, which can transport protein expressed by the vector to the nucleus of a cell.

The vectors described herein can express nucleic acid, e.g. a fusion gene, in a host cell, e.g. a procaryotic or eukaryotic cell. In one embodiment, the vector can be
20 expressed in a bacteria cell, for example, *Escherischia*, e.g. *E. coli*. The nucleic acid in the vector can also be expressed in *Bacillus*. It can also be expressed in baculoviruses, *pichia* expressions systems, and animal tissue or cells, for example insect, mammal, e.g., a human, or yeast (such as *Saccharomyces*). Examples of specific cells include somatic or embryonic cells, HeLa cells, human 293 cells, monkey COS-7 cells, etc.

25 The vector can comprise a number of other components. For example, the vector can comprise a marker, for example a positive or negative selection marker, e.g. ampicillin or kanamycin. The vector can comprise two markers, wherein the first marker is capable of detecting propagation of a vector (e.g. a plasmid) in a bacterial cell

and a second marker is capable of detecting propagation of the vector in a eukaryotic cell. The vector can comprise an origin of replication for bacteria and an origin of replication that is capable of mediating production of a single-stranded DNA by a bacteriophage, such as f1 phage or M13 phage.

- 5 The vector can comprise a promoter. In one embodiment, the promoter is a viral promoter, such as a retroviral or adenoviral promoter. Examples of suitable promoters include T7, lac, trc, tac, CMV, SV40, the cytomegalovirus immediate-early promoter, the retroviral LTR and the TK promoter. In a preferred embodiment, the promoter can be selected for high-level expression. A promoter can be selected for optimal
- 10 expression in bacteria, (e.g. T7, lac, trc, tac etc.) or in a eukaryotic cell (e.g. CMV or SV40).

- The vector can also comprise enhancers, ribosomal binding sites and transcription binding sites. In one embodiment is a vector depicted in Figure 13A, B or C or Figure 14. In one embodiment, a vector comprises HDAG nucleic acid (with or
- 15 without a nuclear local signal), a heterologous gene, a marker, an origin of replication for a host cell, an origin of replication capable of mediating production of single-stranded DNA by a bacteriophage, a promoter, and a ribosome binding site. In one embodiment, the origin of replication is selected for maintaining plasmid expression in *E. coli*.

- 20 Especially preferred is a vector for overexpression of hepatitis delta antigen in *E. coli*, for example, a vector produced by the method as herein described in Example 2, below, e.g. a vector comprising a nucleic acid molecule sequence optimized for expression of HDAG in *E. coli*. The gene can be inserted into a vector which allows production of several forms of the capsid protein including residues 1-84 (terminated in
- 25 the middle domain), the short isoform and the long isoform. In a preferred embodiment, the vector is pR5δV5. Another preferred embodiment is a cassette expression system which allows any expressed sequence or sequences (e.g. a binding moiety) to be appended to the N-terminus of C-terminus of the HDAG octamerization scaffold. In a

preferred embodiment, the HDAg gene is mutated such that in the expressed peptide, a serine residue of HDAg is replaced with (substituted by) a cysteine to allow for convenient chemical cross-linking of the octamerization domain, e.g., to an inert support matrix (e.g. polyethylene glycol), to a synthetic peptide, to an oligosaccharide ,
 5 to a small organic molecule, or to lipids. Figure 12 (A and B) depicts a representation of a construct containing eight appended protein domains on an HDAg octameric framework.

The vector can be viral. Viral vectors include baculovirus, retrovirus, adenovirus, parvovirus (e.g., adeno-associated viruses), coronavirus, negative strand
 10 RNA viruses such as orthomyxovirus (e.g., influenza virus), rhabdovirus (e.g., rabies and vesicular stomatitis virus), paramyxovirus (e.g. measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double stranded DNA viruses including adenovirus, herpesvirus (e.g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e.g., vaccinia, fowlpox and canarypox).
 15 Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus, for example. Examples of retroviruses include: avian leukosis-sarcoma, mammalian C-type, B-type viruses, D-type viruses, HTLV-BLV group, lentivirus, spumavirus. Other examples include murine leukemia viruses, murine sarcoma viruses, mouse mammary tumor virus, bovine leukemia virus, feline leukemia
 20 virus, feline sarcoma virus, avian leukemia virus, human T-cell leukemia virus, baboon endogenous virus, Gibbon ape leukemia virus, Mason Pfizer monkey virus, simian immunodeficiency virus, simian sarcoma virus, Rous sarcoma virus and lentiviruses. Fundamental Virology, Third Edition, edited by B.N. Fields, D.M. Knipe, P.M. Howley, et al. Lippincott-Raven Publishers, Philadelphia (1996) and additional
 25 examples of viruses are described in detail in *Fields Virology*, Third Edition edited by B.N. Fields, D.M. Knipe, P.M. Howley *et al.*, Lippincott-Raven Publishers, Philadelphia, PA (1996).

A nucleic acid molecule described herein can be introduced (incorporated or inserted) into the host cell, by known methods. Such cells, comprising such nucleic acid molecules, are encompassed in the invention. The host cell can be a eukaryotic or prokaryotic cell and includes, for example, baculoviruses, *Pichia* expression systems, yeast (such as *Saccharomyces*), bacteria (such as, *Escherichia* or *Bacillus*), animal cells or tissue, including insect or mammalian cells (such as somatic or embryonic human cells, Chinese hamster ovary cells, HeLa cells, human 293 cells and monkey COS-7 cells, etc.). Examples of suitable methods of transfecting or transforming cells include calcium phosphate precipitation, electroporation, microinjection, infection, lipofection and direct uptake. Methods for preparing such recombinant host cells are described in more detail in Sambrook *et al.*, "Molecular Cloning: A Laboratory Manual," Second Edition (1989) and Ausubel, *et al.* "Current Protocols in Molecular Biology," (1992), for example.

The host cell is then maintained under suitable conditions for expression and recovering the molecule, e.g. a fusion molecule. Generally, the cells are maintained in a suitable buffer and/or growth medium or nutrient source for growth of the cells and expression of the gene product(s). The growth media are not critical to the invention, are generally known in the art and include sources of carbon, nitrogen and sulfur. Examples include Dulbeccos modified eagles media (DMEM), RPMI-1640, M199 and Grace's insect media. Again, the selection of a buffer is not critical to the invention. The pH which can be selected is generally one tolerated by or optimal for growth for the host cell.

The cell is maintained under a suitable temperature and atmosphere. Anaerobic host cells are generally maintained under anaerobic conditions. Alternatively, the host cell is aerobic and the host cell is maintained under atmospheric conditions or other suitable conditions for growth. The temperature should also be selected so that the host cell tolerates the process and can be for example, between about 30° and 40°C for mammalian cells and between 20 and 40°C for bacteria, yeast and insect cells.

The recombinant molecules, including fusion molecules, produced by the processes described herein can be isolated and purified by known means. Examples of suitable purification and isolation processes are generally known and include ammonium sulfate precipitation, dialysis, gel filtration, immunoaffinity, chromatography, electrophoresis, ultrafiltration, microfiltration or diafiltration.

In addition the fusion molecule can incorporate commonly used sequence tags e.g. his, tag or fla to facilitate purification via ligand affinity chromatograph. The fusion molecule is preferably purified substantially prior to use, particularly where the protein will be employed as an *in vivo* therapeutic, although the degree of purity is not necessarily critical where the molecule is to be used *in vitro*. In one embodiment, the bifunctional molecule can be isolated to about 50% purity (by weight), more preferably to about 80% by weight or about 95% by weight. It is most preferred to employ a molecule which is essentially pure (e.g., about 99% by weight or to homogeneity).

Fusion molecules which are prepared according to the above method can be used directly in the disclosed methods or can be screened for an activity prior to use. To screen the fusion molecule for activity, for example, *in vitro*, the fusion molecule (or mixtures of fusion molecules) can be contacted with, for example, the binding partner of a binding moiety of the fusion molecule under conditions suitable for binding and then assayed for binding. For example, a fusion molecule comprising a ligand can be screened for the ability to bind the ligand's receptor, or the binding protein of the ligand's receptor, *in vitro*, by contacting the receptor (or portion thereof) and the fusion molecule under conditions suitable for binding and detecting binding.

METHODS

The HDAG molecules of the invention are useful in a variety of methods. The N-terminal octamer may serve as a convenient high valency framework for linking, presenting or delivering a variety of binding moieties, e.g. as described above. For example, the molecules are useful in the delivery of one or more therapeutic agents,

such as drugs, proteins or polynucleotides (e.g., genes) or products thereof to a patient. The polynucleotide or the product thereof can be a therapeutic agent. In one embodiment, therapeutic polynucleotide includes RNA (e.g., ribozymes) and antisense DNA that prevents or interferes with the expression of an undesired protein in the target cell. The polynucleotide can also encode a heterologous therapeutic protein. A heterologous protein or polynucleotide is one which is not HDAG. Examples of therapeutic proteins include antigens or immunogens such as a polyvalent vaccine, cytokines, tumor necrosis factor, interferons, interleukins, adenosine deaminase, insulin, T-cell receptors, soluble CD4, epidermal growth factor, human growth factor, blood factors, such as Factor VIII, Factor IX, cytochrome b, glucocerebrosidase, ApoE, ApoC, ApoAI, the LDL receptor, negative selection markers or "suicide proteins", such as thymidine kinase (including the HSV, CMV, VZV TK), anti-angiogenic factors, Fc receptors, plasminogen activators, such as t-PA, u-PA and streptokinase, dopamine, MHC, tumor suppressor genes such as p53 and Rb, monoclonal antibodies, antigen binding fragments or constant regions thereof, drug resistance genes, ion channels, such as a calcium channel or a potassium channel, and adrenergic receptors, etc.

Also encompassed by the present invention are the use of HDAG fusion molecules for high through put screening assays, such as for detecting ligand and cell specific receptor binding pairs. The ligand and/or receptor can be peptides (including post-translationally modified proteins) and/or small molecules (including sugars, steroids, lipids, anions or cations). The ligands and ligand-cell specific receptors can be known or unknown. Where the ligand is known and the receptor is unknown, ligand-cell specific receptors can be identified, for example, by screening for host cells transfected with nucleotides encoding potential receptors. For example, the ligands can be secreted (such as chemokines) or non-secreted (such as the extracellular domains of chemokines receptors) proteins.

A library of host cells displaying putative ligand-cell surface receptors can be obtained by transfecting suitable host cells with nucleic acid constructs, including but

not limited to cDNA or genomic libraries, under appropriate regulatory control to result in the expression of cell-surface receptors on the host cell. The fusion molecule with ligand is added to the population of host cells under conditions suitable for introduction. Introduction can be detected, for example, with a label. A similar approach can be used to select unknown ligands in the case where the ligand-cell specific receptors are known and the ligand is unknown. In this embodiment, a library of fusion molecules with putative ligands (e.g., chemokines) can be obtained and contacted with one or more host cells displaying cell surface receptors.

A similar approach can be used to identify unknown ligands, test substances, drugs wherein the cell surface receptor is known, where the fusion molecule comprises a binding moiety which is a receptor which binds a surface molecule. The host cell expresses a distinct ligand or a collection of recombinant ligands. Ligand-receptor binding can be detected following introduction of the molecule to the host cell.

The invention is also particularly useful for vaccine delivery. In this embodiment, an antigen or immunogen can be expressed heterologously (e.g., by recombinant insertion of a nucleic acid sequence which encodes the antigen or immunogen (including antigenic or immunogenic fragments) into a vector comprising HDAG). Alternatively, the antigen or immunogen and HDAG can be expressed in a live attenuated, pseudotyped virus vaccine, for example. Generally, the methods can be used to generate humoral and cellular immune responses, e.g. via expression of heterologous pathogen-derived proteins or fragments thereof in specific target cells.

The dosage administered (e.g., the effective amount) will, of course, vary depending upon known factors such as the pharmacodynamic characteristics of the particular agent, e.g., the therapeutic binding entity, and its mode and route of administration; age, health, and weight of the recipient; nature and extent of symptoms, kind of concurrent treatment, frequency of treatment, and the effect desired.

It can be administered one to several times per day, depending on the mode of administration. Effective doses can be determined by those of skill in the art. An

effective dose of an agent is an amount sufficient to relieve the individual of the symptoms of the disorder which the agent is intended to treat.

Methods of introduction of the agent at the site of treatment include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral, 5 intranasal, gene therapy, cellular implantation or particle bombardment. Other suitable methods include or employ biodegradable devices and slow release polymeric devices.

Because proteins are subject to being digested when administered orally, parenteral administration, e.g., intravenous, subcutaneous, or intramuscular, would ordinarily be used to optimize absorption.

10 For parenteral administration, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. The molecule comprising the agent can be administered in a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle. Examples of such vehicles are 15 water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Liposomes and nonaqueous vehicles such as fixed oils can also be used. The vehicle or lyophilized powder can contain additives that maintain isotonicity (e.g., sodium chloride, mannitol) and chemical stability (e.g., buffers and preservatives). The formulation is sterilized by commonly used techniques. Suitable pharmaceutical 20 carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, A. Osol, a standard reference text in this field of art. Ampules are convenient unit dosages. Formulations for transdermal or transmucosal administration generally include penetrants such as fusidic acid or bile salts in combination with detergents or surface-active agents. The formulation can then be manufactured as aerosols, 25 suppositories, or patches.

Oral agents may be administered if formulated as to be protected from digestive enzymes. If administered orally, the SCR-P will be administered in a therapeutic

composition which may also include an appropriate carrier (e.g., a physiologically compatible carrier), a flavoring agent and a sweetener.

Suitable pharmaceutical carriers include, but are not limited to water, salt solutions, alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrrolidone, etc. The pharmaceutical preparations can be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, coloring, and/or aromatic substances and the like which do not deleteriously react with the active compounds. They can also be combined where desired with other active agents, e.g., enzyme inhibitors, to reduce metabolic degradation.

Using procedures similar to those described above, HDAG molecules (e.g. fusion molecules) and vectors (e.g. cassette expression systems) comprising nucleic acid molecules, such as the vectors described herein, can be used for a variety of purposes. For example, the vector comprising all or part of a nucleic acid sequence of Figure 9 or Figure 15 (synthetic) can be used to overexpress the hepatitis delta antigen in bacteria.

In a preferred embodiment, multiple copies of a binding moiety (e.g. a peptide or domain) can be expressed using the vectors described herein, e.g., by inserting a nucleic acid cassette encoding the moiety into the vector, transforming a host cell with the vector and culturing the cell under conditions sufficient for expression of the moiety. Up to sixteen copies of the moiety (eight at the C terminus of the HDAG monomer and eight at the N terminus) can be made in this way. In a preferred embodiment, the vector is one depicted in a Figure selected from the group consisting of Figure 13A, 13B, 13C and 14.

In one embodiment, a vector or molecule described herein can be used for a high valency expression of a binding moiety, for example, a peptide or protein domain, e.g., an antigen. In another embodiment, the vector can be used to express a high valency

display of at least two different peptides or protein domains, to enhance interaction between ligands. The interaction between ligands can occur in solution, on membranes or on surfaces.

In another embodiment, interaction (e.g., fusion) between two cells (or two cell types) can be mediated or enhanced by, for example, associating an HDAG octamer expressing multiple copies of a domain which interacts with a ligand on the surface of cell type one and multiple copies of a domain which interacts with a ligand on the surface of cell type two. This method could work for embodiments involving more than two cells or cell types as well. A fusion molecule, e.g., an octamer construct of the present invention, which can be coupled to a surface, for example, by chemical cross-linking or by inclusion of at least one copy of a second domain which interacts with a ligand displayed on a surface, can be used to display multiple copies of a domain on a surface. In another embodiment, the octamer can be used to express different enzymes from a linked pathway on a single framework, e.g., for facilitating rapid exchange of substrates and products between enzymes from a single pathway. The enzymes implicated Krebs in the can be cycle.

In another embodiment, the octamer can also be used to link a first binding moiety, e.g. a peptide or domain mediating (specifying) an interaction (an "interaction" domain) and a second binding moiety, which mediates an effect (an "effector" domain). In another embodiment, the effector domain is a chemical, e.g. a drug, linked to the octamer via a free -SH group on the octamer. In a preferred embodiment, the interaction domain mediates interaction with a specific receptor on a cell surface, and the effector domain generates a specific function, such as cell killing.

In another embodiment, the octamer can contain one or more copies of an domain for interaction with a ligand, and one or more copies of a domain which is a label (e.g. alkaline phosphatase, radiolabel, streptodevice, and green fluorescent protein) for amplifying a signal in a solid phase assay, e.g. an ELISA assay.

In another embodiment, an oligomer construct can be used to couple an oligonucleotide which interacts (e.g. hybridizes) with a nucleic acid molecule in the cell (e.g. a specific complementary DNA or RNA sequence) and one or more copies of an effector to target specific DNA or RNA sequences for cleavage. In a preferred embodiment, the effector is a double-stranded nuclease. In a preferred embodiment, the octamer is a mutant with a free sulfhydryl (-SH) group. An octamer construct can be used to couple multiple copies of a ligand in such a way that the interaction of the octamer with a cell triggers signaling or internalization by pathways which depend on the multimerization of a receptor or ligand on the cell surface. The vectors can be used to promote interaction between intracellular components in a signal transduction pathway, for example components which are upstream or downstream from each other.

In another embodiment, the system can be used to mediate efficient (drive) gene expression by coupling (e.g. covalently) an enhancer recognition protein and at least one promoter binding protein.

In another embodiment, the system can be used as a high valency trap to identify the vector can be used as a diagnostic. In one embodiment, the binding moiety is Sp120 and the molecule is used to test for the presence of Human Immunodeficiency Virus. In another embodiment, at least one binding moiety is a drug which has a therapeutic effect when administered to an animal. In another embodiment, a molecule of the present invention is used to screen test substances for an effect. In another embodiment, an agent can be administered to a patient, wherein the agent will inhibit formation of the coiled coil HDAG oligomer. Peptides (e.g. proteins) and nucleic acids, as discussed above, the inventions described herein are based upon the discovery that the HDAG protein oligomerizes to a coiled-coil octamer. The HDAG protein is derived from the Hepatitis D virus which bind to peptides or protein domains.

In another embodiment, at least one binding moiety is a drug which has a therapeutic effect when administered to an animal.

In another embodiment, a molecule of the present invention is used to screen test substances for an effect.

In another embodiment, an agent can be administered to a patient, wherein the agent will inhibit formation of the coiled coil HDAg oligomer.

5 As discussed above, the inventions described herein are based on the discovery that the HDAg protein oligomerizes to a coiled-coil octamer. The HDAg protein is derived from the Hepatitis D virus.

Whereas Hepatitis B virus infection alone generally causes mild, sometimes chronic, hepatitis, coinfection of hepatitis D virus (HDV) with hepatitis B virus (HBV)
10 causes severe, and often fatal, liver disease in humans, and is the most common cause of fulminant viral hepatitis, Hoofnagle, J.H., *J. Am. Med. Assoc.*, 261:1321-1325 (1989). The virus is an obligatory subviral satellite of HBV, requiring the hepatitis B surface antigen (HBsAg) for assembly and cell-to-cell transmission. Rizzetto, M. *et al.*, *Proc. Natl. Acad. Sci. USA*, 77:6124-6128 (1980). However, the viral genome can replicate in
15 the absence of HBV. Kuo, M.Y.P *et al.*, *J. Virol.* 63:1945-1950 (1989). Hepatitis delta encodes all of the information required to direct replication of its RNA genome by the host RNA Pol II. Efficient transmission of hepatitis delta virus requires that the viral RNA and the capsid protein be encapsidated within the hepatitis B virus surface antigen. The viral genome is a 1.7 kilobase single-stranded circular RNA, which is
20 approximately 70% complementary to itself, Wang, K.S. *et al.*, *Nature*, 323:508-513 (1986), and forms a rod-like structure, Kos., A. *et al.*, *Nature*, 323:558-560 (1986). The virus is believed to replicate by a double rolling-circle mechanism in infected cells, Taylor, J., *Cell*, 61:371-373 (1990). Both the genomic and antigenomic strands of the virus contain ribozymes, Wu, H. *et al.*, *Proc. Natl. Acad. Sci. USA* 86:1831-1835
25 (1989), Wu, H.N. *et al.*, *Science* 243:652-654 (1989), Sharmeen, L. *et al.*, *J. Virol.*, 62:2674-2679 (1988), Kuo, M. *et al.*, *J. Virol.* 62:4439-4444, which are responsible for reducing multimeric viral genomes into unit length and for directing the religation of the linear genomes, Sharmeen, L. *et al.*, *J. Virol.* 63:1428-1430 (1989). The antigenomic

strand of the genome encodes the only viral protein known to be associated with HDV, the hepatitis delta antigen (HDAg) (also known as delta virus capsid protein). Wang, K.S. *et al.*, *Nature* 323:508-513 (1986), Makino, S. *et al.* *Nature*, 329:343-346.

HDAg exists in two isoforms. Early in the life cycle of the virus, HDAg is
 5 expressed as a 195-amino acid protein, the small hepatitis delta antigen (s-HDAg), which functions as a transactivator of HDV RNA replication. This form predominates early in infection. Kuo, M.Y.P. *et al.*, *J. Virol.* 63:1945-1950 (1989). Later in the life cycle of the virus, there is an RNA editing event that changes the UAG stop codon of the HDAg-S to a UGG codon, encoding a tryptophan. This allows translation to
 10 proceed for an additional 19 amino acids, resulting in a 214-amino acid residue form of the protein, the large delta antigen (HDAg-L). The 19 amino acids include a stop signal which allows the large isoform to be farnesylated at its terminus. HDAg-L is a potent inhibitor (dominant repressor) of HDV replication, Chao, M. *et al.*, *J. Virol.* 64:5066-5069 (1990), Glenn, J.S. & M. *J. Virol.* 65:2357-2361 (1991), and is also involved in
 15 packaging the viral RNA, Chang, F. L. *et al. Proc. Natl. Acad. Sci. USA* 88:8490-8494 (1991), Wang, C.J. *et al.*, *J. Virol.* 65:6630-6636 (1991), Ryu, W.S., *et al.*, *J. Virol.* 66:2310-2315 (1992), and coencapsidation, i.e., the copackaging of the small antigens into the viral particle. It also directs association with the hepatitis B antigen. Chang, F. L. *et al. Proc. Natl. Acad. Sci. USA* 88:8490-8494 (1991), Ryu, W.S. *et al.*, *J. Virol.*
 20 66:2310-2315 (1992), Chang, M.F. *et al.*, *J. Virol.*, 68:646-653 (1994). Both the large and small antigens are highly specific RNA-binding phosphoproteins. Chang, M.F. *et al.*, *J. Virol.* 62:2403-2410, Lin, J.H. *et al.*, *J. Virol.* 64:4051-4058 (1990) and have been shown to recognize specifically the viral rod-like structure of the HDV viral genomes, Chao, M. *et al.*, *J. Virol.* 65:4057-4062 (1991). Crosslinking studies have shown that
 25 both proteins can exist as either homomultimers (all small antigen or all large antigen) or as heteromultimeric structures (a mixture of small and large antigen) Xia, Y.P. & Lai, M.M.C., *J. Virol.* 66:6641-6848 (1992), Wang, J.G. & Lemon, S.M., *J. Virol.* 67:446-454 (1993), Chang, M.F. *et al.*, *J. Virol.* 67:2529-2536 (1993).

There have been a number of structure-function studies of both the large and small delta antigens. The N-terminal third of the small delta antigen contains a putative coiled-coil sequence, Xia, Y.P. & Lai, M.M.C., *J. Virol.* 66:6641-6848 (1992), Wang, J.G. & Lemon, S.M., *J. Virol.* 67:446-454 (1993), Chang, M.F. *et al.*, *J. Virol.* 67:2529-2536 (1993), comprising heptad repeats, which is followed by a linker domain which contains bipartite nuclear localization signal. Xia, Y.P. *et al.*, *J. Virol.* 66:914-921 (1992). The middle portion of HDAG contains two arginine-rich motifs that have been shown to bind to the viral RNA. Lee, C.Z. *et al.*, *J. Virol.*, 67:2221-2227 (1993). The C-terminal segment of s-HDAG is proline- and glycine-rich. Lazinski, D.W. & Taylor, J.M. *J. Virol.*, 67:2672-2680 (1993). L-HDAG is prenylated at the extreme C terminus and it is believed that this part of the molecule interacts with HBsAg and the membranes of the endoplasmic reticulum. Hwang, S.B. & Lai, M.M.C. *J. Virol.*, 67:7659-7662 (1993), de Bruin, W. *et al.*, *Virus. Res.* 31:27-37 (1994). There is also some evidence that common segments of the large and small antigens may have subtly different conformations. Hwang, S.B. & Lai, M.M.C. *Virology* 193:924-931 (1993), Hwang S.B. & Lai, MMC, *J. Virol.* 68:2958-2964 (1994).

The coiled-coil domain has been shown to be required for a number of the functions of both small and large delta antigens. Mutations that destroy or alter the coiled-coil domain either greatly reduce or totally eliminate the ability of the HDAG-S to function as a *trans* activator of replication, Chang, M.F. *et al.*, *J. Virol.*, 68:646-653 (1994), Chang, M.F. *et al.*, *J. Virol.* 62:2403-2410 (1998), Lin, J.H. *et al.*, *J. Virol.* 64:4051-4058 (1990), Chao, M. *et al.*, *J. Virol.* 65:4057-4062 (1991), Xia, Y.P. *et al.*, *J. Virol.*, 66:6641-6648 (1992). These same mutations also prevent the HDAG-L from inhibiting HDV RNA replication and inhibit its function in mediating the copackaging of the small antigen, Chang, M.F. *et al.*, *J. Virol.*, 68:646-653 (1994), Chang, M.F. *et al.*, *J. Virol.* 62:2403-2410, Lin, J.H. *et al.*, *J. Virol.* 64:4051-4058 (1990), Chao, M. *et al.*, *J. Virol.* 65:4057-4062 (1991). Transfection of cells undergoing HDV replication with a plasmid containing just the N-terminal one-third of the delta antigen (which

contains the coiled-coil domain) inhibited HDV replication, Xia, Y.P. & Lai, M.M.C., *J. Virol.*, 66:6641-6648 (1992). However, removal of the coiled-coil domain does not prevent the delta antigen from binding the viral RNA, Lin, J.H. *et al.*, *J. Virol.*, 64:4051-4058 (1990) nor does it prevent the HDAg-L from packaging the viral RNA, Chen, P.J., *et al.*, *J. Virol.*, 66:2853-2859 (1992). A "black sheep" model has been proposed for the mechanism of inhibition of the HDV replication. HDAg-L is believed to disrupt the homo-oligomeric small antigen multimers, essentially poisoning the HDAg-S complex. Xia, Y.P. & Lai, M.M.C., *J. Virol.*, 66:6641-6648 (1992). While the precise role of HDAg-S in replication of HDV is unknown, the protein is not a polymerase, and RNA amplification is thought to be mediated by host cell RNA polymerase II, MacNaughton, T.G. *et al.*, *Virology* 184:387-390 (1991), Fu., T. B. & Taylor, J. *et al.*, *J. Virol.* 67:6965-6972 (1993).

Biophysical studies were undertaken to examine the coiled-coil domain of HDAg. Rozzelle, J.E., Jr. *et al.*, *Proc. Natl. Acad. USA*, 92:382-386 (1995). As described in Example 1, a peptide was synthesized that corresponded to residues 12 to 60 of the δ 12-60(Y). This region includes the N-terminal heptad repeats. The peptide also included a C-terminal tyrosine so that the peptide could be labeled with I^{125} for use in a radioimmunoassay. The peptide sequence was conceptually divided into three segments based on the presence of two potential helix breakers Gly23 (G23) and Pro49 (P49); segments A (residues 12-24), B (residues 25-49), and C (residues 50-60) (Figure 1). The full-length peptide δ 12-60(Y) and two shorter peptides that corresponded to regions A+B and B+C were synthesized. A number of biophysical experiments, including circular dichroism (CD), mass spectrometry, and analytical ultracentrifugation, clearly showed that the δ 12-60(Y) peptide was largely helical and formed a coiled coil Rozzelle, J.E., Jr. *et al.*, *Proc. Natl. Acad. USA*, 92:382-386 (1995). The shorter peptides formed much less stable structures and were considerably less helical than δ 12-60(Y). Human polyclonal antibodies from hemophilic patients who were chronic carriers of HBV and HDV reacted with the δ 12-60(Y) peptide, in both an

ELISA and in a sandwich radioimmunoassay. Rozzelle, J.E., Jr. *et al.*, *Proc. Natl. Acad. USA*, 92:382-386 (1995), Wang, J. G. *et al.* *J. Virol.* 64:1108-1116. Subsequent studies indicated that monoclonal antibodies against the peptide recognized a conformational epitope only presented by the full-length peptide and not the shorter,
 5 extensively overlapping peptides, Rozzelle, J.E., Jr. *et al.*, *Proc. Natl. Acad. USA*, 92:382-386 (1995).

Described herein for the first time is the crystal structure of the peptide $\delta 12-60(Y)$ to 1.8Å resolution. The structure reveals that the capsid protein dimerizes as an unusual antiparallel coiled coil. In the crystal structure, the dimers further
 10 oligomerize to form an octamer. The octamer forms an open, square planar structure with an antiparallel dimer forming each side of the square. Crosslinking and hydrodynamic studies suggest that both the peptide and the full-length short isoform exist as stable octamers in solution.

The structure of the peptide lends new insights into the mechanism by which
 15 HDAG dimerizes and further associates into higher ordered structures. The structure also explains why residues C-terminal to the predicted coiled-coil domain, and the helix-breaking proline residues are important for the stabilization of the coiled-coil structure. The peptide structure has important consequences for the *in vivo* oligomerization of HDAG. The unique octameric structure which is observed in the
 20 crystal structure also suggests that the N-terminus of the molecule may have a previously undetermined function.

When the HDAG open reading frame was originally examined, amino acids from residue 13 to 47 were identified as possibly forming a coiled coil. Glutaraldehyde cross-linking studies of full-length HDAG, as well as of the peptide, confirmed the
 25 formation of dimers, tetramers and higher-ordered structures, Wang, J. G. & Lemon, S.M., *J. Virol.*, 67:446-454 (1993), Rozzelle, J.E., Jr. *et al.*, *Proc. Natl. Acad. USA*, 92:382-386 (1995). The crystal structure of the peptide clearly shows how monomers come together to form antiparallel dimers as well as a higher-ordered octameric

structure. The structure of δ 12-60(Y) also agrees well with previous circular dichroism studies of the peptide, which indicated that the two ends of the peptide (regions A and C) were important for the structural stability of the coiled coil. Rozzelle, J.E., Jr. *et al.*, *Proc. Natl. Acad. USA*, 92:382-386 (1995). Shorter synthesized peptides that were

5 missing either the A or C regions (A+B and B+C), were significantly less helical than the full-length peptide (A+B+C; 37%, 45% and 84% respectively at 37°C). The peptide structure shows that hydrophobic residues from the N terminus of one monomer (region A), not involved in the heptad repeat, interact with residues outside of the predicted coiled-coil domain near the C terminus of the other monomer (region C) to form a

10 hydrophobic core Trp20 (W20), Leu24 (L24), Trp50 (W50), Leu51 (L51) sandwiched between Arg13 (R13) and Arg24 (R24). This may stabilize the structure by keeping the ends of the helix from fraying. An additional stabilizing feature is a hydrogen bond between the sidechain of Glu45 (E45) and the indole nitrogen of Trp20 (W20). These hydrophobic residues, as well as the glutamic acid residue, are highly conserved in the

15 10 different strains of HDV identified to date (Figure 1). In fact, they are more conserved than those residues in the heptad repeat making up the hydrophobic core of the long helix (Figure 1).

As described in Example 3, cross-linking studies of full-length recombinant small delta antigen (r-HDAg-S or r- δ Ag-S) also demonstrated that the recombinant

20 protein forms octamers in solution. This indicates that the octamer form seen in the crystal may not be an artifact of crystallization, but rather may represent the true state of the oligomerization of the delta antigen. A study by Chang and colleagues found that a deletion in the HDAg-L, just C terminal to the coiled-coil domain (residues 50 to 75), prevented the HDAg-S from being copackaged with the HDAg-L, Chang, M.F. *J.*

25 *Viro.*, 66:6019-6027 (1992). HDAg-L with this same deletion could not inhibit HDV replication, whereas a deletion in L-DHAg of residues 65 to 75 could. This suggested that the coiled-coil domain alone is not sufficient for the interaction between the large and small antigens, and that a subdomain between residues 50 and 65 is also necessary

for this interaction. The crystal structure of $\delta 12-60(Y)$ indeed shows the importance of residues between 50 and 60 in the formation of the peptide oligomer. They are not only involved in stabilizing the $\delta 12-60(Y)$ dimer Trp50 (W50) and Leu51 (L51) but are also involved in the formation of the dimer-dimer interface Trp50 (W50), Ile54 (I54), and
 5 Ile58 (I58).

Prior to the studies described here, the overall organization of the HDAG oligomer was unknown. The structure of the $\delta 12-60(Y)$ peptide suggests a number of interesting considerations about the function of the coiled-coil domain of the hepatitis delta antigen. For example, Lai and coworkers, Xia, Y.P., *et al.*, *J. Virol.* 66:6641-6648
 10 (1992), inferring from previous data that showed that as little as 12% of HDAG-L is needed to inhibit 90% of viral activity, Chao, M., *et al.*, *J. Virol.*, 64:5066-5069 (1990), proposed that as little as one part of HDAG-L in eight parts of HDAG-S could inhibit viral replication. Their "black sheep model" proposed that the HDAG-L either disrupted the conformation of the oligomer of HDAG-S, therefore preventing it from binding to
 15 host factors, or that the presence of HDAG-L in the complex prevents the complex from interacting with host factors. This would seem in agreement with the peptide structure of octameric $\delta 12-60(Y)$ and the results of the MALDI-TOF mass spectrometry analysis. If HDAG-L does disrupt the conformation of the oligomer of HDAG-S it probably does not do so directly through the multimerization domain, given that the
 20 large and small delta antigen share the same sequence within this region. Rather, it is possible that this α_7 or $\alpha_n\beta_m$ structure can no longer interact with host factors. Also, since the C terminus of the L-HDAG interacts with the endoplasmic reticulum (ER) membrane and with HBsAg for assembly, it could redirect the complex elsewhere in the cell, preventing the nuclear translocation of s-HDAG which is required for HDV
 25 replication.

Discovery of the organizational structure also provides information regarding possible undetermined functions of the N terminus. The octamer that is formed by the peptide is reminiscent of proteins that form clamps around DNA, such as PCNA.

Talluru, S.R., *et al.*, *Cell* 79:1233-1243 (1994). The 50 Å hole formed by the octameric structure is lined with basic side chains, suggesting that the N terminus of the protein not only may act as a dimerization/oligomerization domain, but also that it may function either as a clamp around the viral RNA or other nucleic acid or perhaps even function as a spool for nucleic acid. There is a report that peptides corresponding to the extreme N-terminal portion of the HDAg residues 2 to 27 and 2 to 17 can bind the viral RNA Poisson, F., *et al.*, *J. Gen. Virol.* 74:2473-2478 (1993), Poisson, F. *et al.*, *J. Virol Methods* 55:381-389 (1995). Since the δ 12-60(Y) structure is missing residues 2 to 11, it is impossible to say what role they play in binding the viral RNA. Of the remaining residues, only Lys25 (K25) and Lys26 (K26), which point into the hole of the octamer, seem likely to play a role in binding RNA by potentially binding the phosphate backbone of the viral RNA.

The large size of the hole may be necessary to accommodate the viral RNA which is only 70% self complementary, and would possess a number of regions of bulged out single-stranded sequence, increasing the radius of gyration of the RNA as well as bending the RNA. Lilley, D.M.J., *Proc. Natl Acad. Sci. USA*, 92:7140-7142 (1995). The octameric structure also implies that there may be as many as four RNA-binding domains on each side of the octamer. This portion of the molecule may also bind another protein, especially one that is acidic, such as the recently discovered delta antigen interacting protein A (dipA), a cellular protein which has been found to interact with the HDAg Brazas, R. *et al.*, *Science* 274:90-94 (1996) and, based on its amino acid sequence, would have an isoelectric point of 4.9.

Many investigators have referred to the putative coiled-coil domain of the delta antigen as a leucine zipper-like region. Experiments involving mutations in this region were interpreted assuming the coiled-coil domain of the delta antigen would resemble the parallel coiled-coil of the bZIP family of transcription factors, such as GCN4. HDAg dimerizes through an antiparallel coiled-coil domain, rather than a standard parallel coiled coil.

Although algorithms have been designed to determine the oligomerization state of a coiled coil, Woolfson, D.N., *et al.*, *Protein Sci.* 4:1596-1607 (1995), Wolf, E., *et al.*, *Protein Sci.* 6:1179-1189 (1997), they cannot determine the orientation of the predicted coiled coil. The discovery that this region forms an antiparallel coiled coil demonstrates that additional biochemical or genetic evidence, such as provided herein, is necessary to determine whether a predicted coiled-coil domain adopts a parallel or antiparallel conformation. Along with the structure of the δ 12-60(Y) peptide, there are other examples of molecules that dimerize through antiparallel coiled-coil domains, such as the *Escherichia coli* regulatory protein AraC, Soisson, S.M., *et al.*, *Science* 276:421-425 (1997) and the replication terminator protein from *Bacillus subtilis*, Bussiere, D.E. *et al.*, *Cell* 80:651-660 (1995).

The hepatitis delta antigen (HDAg), the sole protein made by the hepatitis delta virus (HDV), is essential for viral replication *in vivo*. Oligomerization of the protein is necessary for both the transactivating function of the small delta antigen (HDAg-S) and the *trans* dominant inhibitory effect of the large delta antigen (HDAg-L). The structure of the peptide δ 12-60(Y) that corresponds to the predicted coiled-coil domain of the hepatitis delta antigen HDAg suggests that delta antigen HDAg not only dimerizes through an antiparallel coiled coil, but also forms octamers. Interestingly, the coiled coil is stabilized by hydrophobic residues C terminal to the coiled-coil domain. These C-terminal residues interact with hydrophobic residues in the N terminus of the coiled-coil region. The hydrophobic core of the dimer is extended by further hydrophobic interactions at the interface between dimers in the octameric structure. In contrast to the rather promiscuous interactions between the coiled-coil domain, these unique interactions at the termini of the monomer and dimer interfaces might provide a good target for antivirals against HDV, since disruption of oligomerization can prevent replication *in vivo*.

The surprising octameric structure of the peptide suggests that the capsid of the delta antigen (HDAg) will look very different from the known structures of other viral

nucleocapsid proteins. The octameric structure also suggests important implications for binding of HDAG to the viral RNA, since as many as four of the arginine-rich RNA-binding domains might be needed for binding to the viral RNA. The very basic hole in the octamer suggests that this portion of the molecule may act as a sort of "clamp"

5 around an acidic molecule, such as viral RNA, another nucleic acid or a cellular factor.

The exact function of HDAG in viral replication is unclear. The protein may only function as a shuttle, binding to the viral RNA and transporting it into the nucleus of the infected cell. It is possible that HDAG functions to recruit host cell transcriptional machinery to the viral RNA. The discovery of the structure enables the design of

10 experiments to determine whether the N terminus of the molecule has RNA-binding capabilities and investigate the mechanism of oligomerization and inhibition of small antigen by the large antigen. A systematic examination of the amino acids involved in dimerization and oligomerization would allow the determination of the mechanism by which HDAG-L inhibits HDAG-S. Furthermore, the unique interactions at the termini of
15 the coiled-coil region provide a new framework to be exploited in the *de novo* design of stable antiparallel coiled coils.

The examples presented below are provided as further guidance and are not to be construed as limiting the invention in any way.

EXAMPLE 1: SYNTHESIS OF δ 12-60(Y) PEPTIDE

20 MATERIALS AND METHODS

PEPTIDE SYNTHESIS: The δ 12-60(Y) peptide was obtained by Erickson, B. and Lemon, S.M. and was synthesized and purified as described previously in Rozzelle, J.E. *et al.*, *Proc. Natl. Acad. Sci. USA*, 92:382-386 (1995), incorporated herein by reference in its entirety.

25 The peptide (Fig.11B) was assembled by fluorenylmethoxycarbonyl chemistry and purified by reversed-phase HPLC. It was N $_{\alpha}$ -acetylated and C $_{\alpha}$ -amidated. Crude

peptide in 0.05% trifluoroacetic acid was separated on an octyl-silica column [C₈, Applied Biosystems, 250 mm x 10 mm (i.d.), 300-Å pore size] by elution at 3 ml/min over 50 min with a linear gradient of 20-42% acetonitrile in 0.05% trifluoroacetic acid. Peptide δ 12-60(Y) was eluted at 36% acetonitrile (monitored at 230 nm). The

5 homogeneity of the individual fractions was determined on an analytical octyl-silica column. The expected mass of the peptide was confirmed by electrospray ionization (ESI) mass spectrometry: peptide δ 12-60(Y), m/z 6034.1 \pm 1.2 (calcd. 6033.7).

The peptide from the 12-60 region of HDAG was synthesized (Fig. 11B). Peptide δ 12-60(Y) included segments A, B, and C. Segment B contains three heptads in which the

10 first and fourth heptad positions are occupied by five leucines and one isoleucine, and is probably part of an α -helical coiled coil. A tyrosine residue, (Y), was added, Lys⁶⁰, to the C terminus of δ 12-60(Y) to permit radioiodination.

CD SPECTROSCOPY. The α -helicity and the temperature at the midpoint of thermal denaturation (T_m) of the peptides were determined by CD spectroscopy. All three

15 peptides had high α -helicity in PBS at 5°C. The ratio of the mean residue ellipticity of the negative bands near 222 nm and 208 nm ($[\theta_{222}]/[\theta_{208}]$) is an indicator of coiled-coil formation. Values close to 1.0 indicate an α -helical coiled coil and values near 0.8 indicate isolated α -helices. At 5°C, this ratio was 0.98 for δ 12-60(Y). At 37°C, this ratio was 0.94 for δ 12-60(Y), consistent with persistence of a coiled-coil structure. In contrast, at 37°C

20 this ratio was only 0.79 for δ 12-49 and 0.76 for δ 25-60(Y), inconsistent with a coiled-coil structure.

EXAMPLE 2: SYNTHETIC GENE FOR OPTIMIZED EXPRESSION OF HDAG-S

MATERIALS AND METHODS

EXPRESSION PLASMIDS: pR5 δ V5 was constructed for the high-level expression of

25 HDAG-S in *Escherichia coli*. The protein sequence of the American strain with the HDAG-S (GenBank accession no. M28267) was back-translated with the program BACKTRANSLATE. (This program was from the Wisconsin Package, versions 9.0

[Genetics Computer Group, Madison, Wis.], with *E. coli* codon frequencies obtained from [With the sequence obtained as shown in Figure 9 and Figure 18, the plasmid pR5 \$\delta\$ V5 was constructed by a two-step PCR method, as described previously.](http://gopher://weeds.mgh.harvard.edu:70/Ofnp%3Aweeds.mgh.harvard.edu@/pub/codon/eco.cod.)

- 5 Casimuro, D.R. et al., *Biochemistry*, 26:6640-6648(1995), with the exception that Vent polymerase (New England BioLabs) was used instead of *Taq* polymerase. Eight overlapping synthetic primers were synthesized (Figure 9 and Figure 18). Changes in the back-translated sequences were made so that the overlaps of the PCR primers would have approximately the same melting temperature. Primers were electrophoresed into a
- 10 10% sequencing gel, visualized by UV shadowing, and excised from the gel. The primers were then purified with a Waters Sep-Pak column.

- The first PCR contained 4 pmol of each of the eight primers in a 100- μ l reaction mixture. Ten microliters of the first PCR was added to a second reaction mixture that contained an upstream primer (5'-GGGCATATGAGCCGTAGCGA) and a downstream
- 15 (5'-GCGCCATGGTTTACGGAAAG) primer designed to amplify the desired full-length product. Both reactions involved a hot start at 94°C followed by 30 cycles of 1 min. at 94°C, 1 min at 57°C, and 1 min at 72°C, with a final 5-min extension at 72°C.

- The PCR product from the second reaction was cloned into the vector pCR-
- 20 Blunt (Invitrogen), which allows selection based on disruption of a toxic gene. Plasmids isolated from colonies were checked for the insert by restriction digest mapping. The open reading frame of HD δ Ag-S was subcloned into expression vector pRSETb (Invitrogen). The sequence of the resultant plasmid, pR5 δ V5, was verified by dye termination sequencing.

- 25 **PROTEIN PURIFICATION:** Recombinant HD δ Ag-S (δ Ag-S) was expressed and purified as follows. Plasmid pR5 δ V5 was transformed into BL21(DE3)pLysS cells (Novagen). A single colony was used to inoculate a 100-ml overnight culture. Ten milliliters of this overnight culture was used to inoculate a 1-liter culture. At an optical

density of between 0.4 and 0.6, the cells were induced with 3 ml of 100 mM IPTG (isopropyl- β -D-thiogalactopyranoside). Cell growth was continued for 3 h, and then cells were pelleted at 5,000 x g for 10 min. The cells were resuspended in 15 ml of 50 mM HEPES (pH 7.5) - 250 mM NaCl - 1 mM $MgCl_2$ and stored at -20°C until needed.

5 The frozen cells (45 ml corresponding to three 1-liter cultures) were thawed, and one Complete Protease Inhibitor tablet (Boehringer Mannheim) was added, along with RNase A and DNase I, to a final concentration of 50 μ g/ml. Cells were lysed by sonication and pelleted at 10,000 x g for 30 min. The lysate was diluted threefold with 50 mM HEPES buffer (pH 7.5) and then applied to a 10 x 1.5-cm Fast SP Sepharose
10 column (Pharmacia) equilibrated with 50 mM HEPES buffer (pH 7.5) and eluted with a salt gradient from 0 to 1 M NaCl in 50 mM HEPES (pH 7.5). The fractions containing δ Ag-S were applied to a Superdex S-200 column (Pharmacia) equilibrated with 50 mM HEPES (pH 7.5), 500 mM NaCl, and 5% glycerol. The HDAg-S obtained was >85% pure as judged by Coomassie blue staining of a sodium dodecyl sulfate gel.

15 Proteins with a histidine tag were purified as follows. Proteins expressed in *E. coli* were affinity purified with a Talon column according to the recommendations of the manufacturer (Clontech). Proteins expressed in mammalian cells were purified by the Invitrogen Xpress System. In both cases, the fractions containing the purified protein were identified by sodium dodecyl sulfate gel electrophoresis, pooled, dialyzed,
20 and concentrated.

TRANSFECTION: Plastic 16-mm-diameter tissue culture wells (Costar) were seeded with approximately 0.1×10^6 Huh7 cells, Nakabayoshi, H. *et al. Cancer Res.*, 42:3858-3863 (1982). For transfections with assembled RNP, 0.25 to 900 ng of HDAg-S and 500 ng of genomic HDV RNA in 125 μ l of Opti-MEM were combined with 2.7 μ l of
25 lipofectamine (2 mg/ml) in 125 μ l of Opti-MEM, incubated for 30 min at room temperature, and applied to cells that had been washed with Opti-MEM (Hawley-Nelson, P. *et al., Focus*, 15:73-79(1993)). In control transfections, either HDAg-S or HDV RNA was omitted. For cDNA transfections, 500 ng of plasmid DNA was used.

At 5 h after transfection, the transfection mixture was changed to Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. At 4 days after transfection, cells were reseeded into a 30-mm-diameter dish containing a glass coverslip; at 8 days, the cells were examined by immunofluorescence microscopy. Eight days length was
5 chosen for three reasons: (i) to avoid detection of the transfected HDAG-S; (ii) In the immunofluorescence assays, 8 days corresponded to the peak signal for a cell undergoing RNP-initiated replication; (iii) At 8 days, HDAG-L, created as a consequence of both RNA editing and genome replication, could be readily detected (Luo *et al. J. Virol.*, 64:1021-1027 (1990)). In contrast, for Northern analyses, genome replication
10 was detectable as early as 2 days.

RESULTS

Initial studies with *E. coli* demonstrated poor expression of HDAG-S from the wild-type sequence. About 18% of the codons in the natural HDAG sequence are rarely used by *E. coli*. Attempted overexpression of codons that are rare in *E. coli* not only
15 can inhibit expression but also can lead to misincorporation (Del Tito, B.J. *et al., J. Bacterial*, 177:7086-7091 (1995) Therefore, a nucleotide sequence was designed which maintained the amino acid sequence, but increased the percentage of codons that were most favored for expression in *E. coli* from 26% to 85%. This optimized sequence (Figure 9) was used to construct expression plasmid pR5δV5. Thus, a 40-fold increase
20 in expression was obtained and the recombinant protein was purified to > 85% homogeneity.

EXAMPLE 3: STRUCTURE DETERMINATION

MATERIALS AND METHODS

CRYSTALLIZATION AND DATA COLLECTION: The peptide $\delta 12-60(Y)$ was dissolved in 50mM acetate, pH 4.8, 50 mM NaCl and brought to a concentration of 15 mg/ml. The crystals of the $\delta 12-60(Y)$ peptide were grown at 22°C by the vapor diffusion method. The peptide (2 μ l of a 15 mg/ml solution) was mixed with 2 μ l of the reservoir solution containing 100 mM sodium acetate, pH 4.8, and 100 mM sodium citrate, pH 5.6, on a coverslip and then inverted over the reservoir solution. Crystals appeared within 3-4 days, and grew as large as 0.5 x 0.3 x 0.3 mm. Crystals belonged to space group $P2_12_12$ with unit cell parameters $a=109.2$ Å, $b=85.3$ Å, $c=29.4$ Å, $\alpha = \beta = \gamma = 90^\circ$. When attempts to find a heavy atom derivative failed, a peptide was synthesized with serine 22 replaced by a cysteine, $\delta S22C12-60(Y)$. The $\delta S22C12-60(Y)$ peptide was reacted with an excess of platinum terpyridine, dialyzed overnight against water, and then freeze-dried. The peptide was then reconstituted at 15 mg/ml in 50 mM acetate, 50 mM NaCl, 5 mM DTT, pH 4.8, and crystallized by the same conditions as that of the wild type-peptide. This peptide crystallized isomorphously with the $\delta 12-60(Y)$.

The coverslips containing the crystals were inverted and cryosolvent (reservoir solution containing 30% glycerol) was slowly mixed with the drops and continuously replaced until no mixing was observed. The crystals were mounted in nylon loops and frozen directly in the nitrogen stream. Crystals used at Brookhaven were stored in liquid nitrogen until the time of data collection. Two native data sets were collected at Beamline X12C at the National Synchrotron Light Source at Brookhaven National Lab using X-rays of wavelength 1.15Å (Table 1). The heavy atom data set was collected on a Siemens rotating anode with a multiwire detector (Table 1). Data from the native crystals was processed using DENZO, Otwinowski, Z. SERC Daresbury Laboratory, Warrington, UK:(56-62 (1993)) and SCALEPACK. Data from the heavy atom

derivative was integrated using the program BUDDHA (Blum, M. *et al.*, *J. Appl. Cryst.* 20:235-242 (1987)) and processed using ROTAVATA and AGROVATA from the CCP4 package (CPP4 *Acta. Cryst. D.*, 50:760-763 (1994)). Structure factors from both data sets were calculated using TRUNCATE (CPP4, *supra*). Data from the native and
5 derivative were scaled together using SCALEIT (CPP4, *supra*).

STRUCTURE DETERMINATION AND MODEL BUILDING: The positions of the heavy atom sites were determined using SHELXS-86 (Sheldrick, G.M., *Acta. Cryst. A.* 46:467-473 (1990)). The positions of the heavy atom sites were refined using MLPHARE (Otwinowski, Z., *Proceedings of the CCP4 Study Weekend*, 80-86 SERC
10 Daresbury Laboratory, Warrington, UK (1991)), and initial SIRAS phases were calculated. The data was then subjected to a round of solvent flattening with histogram matching using DM (Zhang, K.Y.J. & Main, P. *Acta. Cryst. A.* 46:41-46 (1990). A map was calculated which clearly showed the position of the two dimers in the asymmetric unit, and an initial model was built into the initial SIRAS map using the program O
15 (Jones, T.A. *et al. Acta Cryst. A.*, 47:110-119 (1990)). The structure was refined using X-PLOR 3.8.9, Brünger, A.T., *Yale University Press*, New Haven, CT (1992). Rounds of positional refinement, followed by simulated annealing and B-factor refinement, were carried out with rebuilding of the structure using O between cycles of refinement. During the initial model building and refinement, omit maps, which excluded 10
20 residues at a time, were used to check the progress of refinement.

SURFACE AND ELECTROSTATIC CALCULATIONS: Surface calculations were performed using the surface option in QUANTA version 4.0. Electrostatic calculations were performed with GRASP version 1.3.

PROTEIN EXPRESSION AND PURIFICATION: The pR5δV5 plasmid, Dingle, K. *et al. J. Virol.*, 72(6):4783-4788 (1998) which contains a synthetic gene for the small delta antigen, HDAG-S, was transformed into BL21 (DE3)pLysS cells (Novagen) and purified as described previously in Example 2. See also Dingle, K. *et al.*, *supra*.

- 5 Briefly, 45 ml of frozen cells, corresponding to three 1 L cultures, were thawed and one protease inhibitor tablet (Boehringer Mannheim) was added, as well as RNase A and DNase I to a final concentration of 50 µg/ml. Cells lysed by sonication were pelleted at 10,000 x g for 30 minutes. The lysate was diluted three-fold with 50mM HEPES buffer, pH 7.5, and then applied to a 10 x 1.5 cm Fast SP Sepharose (Pharmacia)
- 10 column equilibrated with 50 mM HEPES buffer, pH 7.5, and eluted using a salt gradient from 0 - 1M NaCl in 50mM HEPES, pH 7.5. The fractions containing recombinant small delta antigen (rδAg-S [r-HDAG-S]) were assayed using SDS-PAGE and pooled. The sample was then applied to a Superdex S-200 column (Pharmacia) equilibrated with 50 mM Hepes, pH 7.5, 500 mM NaCl and 5% glycerol. The elution of the protein from
- 15 the column was monitored by UV absorbance at 280 nm.

RESULTS

- Attempts to find a heavy atom derivative using the peptide with the wild-type sequence of the American strain of HDAG failed. Thus, a new peptide was synthesized with a cysteine replacing serine 22 (Ser22) (this residue demonstrates considerable
- 20 variation in different strains of HDV, Figure 1). The cysteine mutant and wild-type peptides crystallized isomorphously. The presence of cysteine 22 (Cys22) allowed the preparation of a platinum terpyridine derivative, facilitating the determination of the structure using SIRAS methods (Table 1). Retrospective examination of the model confirmed that the Pt was bound to the sulfur of cysteine 22 (Cys22).

- 25 The solvent-flattened map was easily interpretable, and clearly showed two dimers in the asymmetric unit. Rounds of positional refinement, simulated annealing, temperature factor refinement using X-PLOR (Brünger, A.T., *Yale University Press*,

New Haven, CT (1992)), and manual rebuilding using O (Jones, T.A. *et al.*, *Acta Cryst. A*, 47:110-19 (1990)), led to the current model (Table 2, Figure 2). The current model has an R factor of 22.5% and a free R factor of 27% with good geometry (r.m.s.d. bond 0.007 Å and r.m.s.d. bond angles 1.0°). A number of sidechains exposed to the large solvent channel, as well as the first residue in the chain and the last residue in one of the chains, are disordered. The four monomers in the asymmetric unit superimpose well onto one another, with an average r.m.s.d. for mainchain atoms of 0.81 Å and for all non-hydrogen atoms 1.51 Å. The main differences in the monomers are those residues involved in crystal packing interactions.

10 The coordinates have been deposited in the Brookhaven Protein Data Bank (accession number 1A92).

Each monomer is composed of a long, N-terminal helix, approximately 60 Å in length, interrupted by a sharp bend at proline 49 (Pro49), and continuing on into another short helix. The long helices of each of two monomers wrap around each other forming an antiparallel coiled coil (Figure 3a, Figure 3b), which straightens out at the N terminus. Only one of the four possible salt bridges between Glu31 (E31) and Lys38 (K38) is seen. In the other three cases, the charged groups are slightly farther apart (3.8 Å, 4.2 Å and 4.4 Å versus 2.9 Å) and the sidechains are hydrogen bonded to nearby solvent molecules. The sidechain of Glu45 (E45) is hydrogen bonded to the indole nitrogen of Trp20 (W20). The sidechain of Asn48 (N48), which is located at the C terminus of the long helix, completes the hydrogen-bonding pattern of the helix by making a hydrogen bond back to the mainchain oxygen of Leu44 (L44). The formation of the dimer buries 2650 Å² of surface area, approximately 26% of the total surface area.

Although the majority of residues in the heptad repeat (Figure 3c) of the predicted coiled-coil region do pack as expected, Trp20 (W20) does not. Even though the Cα-Cβ vector of Trp20 (W20) points out of the interface as would be expected for a sidechain in the *a* position of a heptad repeat, the sidechain of Trp20 is flipped away from the core of the coiled coil and into a hydrophobic region formed between segment

A (residues 12-24) of one monomer, and segment C (50-60) of its partner within the peptide dimer. The dimer shows primarily hydrophobic interactions between residues in the A and C regions. Ile16 (I16), Leu17 (L17), Trp20 (W20), Trp50 (W50), and Leu51 (L51) are the sidechains primarily involved in this hydrophobic region, which is capped by the aliphatic portion of the sidechains of Arg13 (R13) and Arg24 (R24) (Figure 4). The primary non-hydrophobic, monomer-monomer interactions near this region involve the formation of a hydrogen bond between Trp20 (W20) and Glu45 (E45) (Figure 4). The heptad repeat is also unusual in that it contains a glycine at position 23. If the monomers were oriented in a parallel fashion, a large hole in the middle of the hydrophobic core of the dimer would result. However, since the strands are arranged antiparallel, the large sidechain of Ile41 (I41) packs into the hole formed by Gly23 (G23). The dimer is stabilized by hydrophobic interactions other than the residues in the heptad repeat. Residues from the N-termini of each monomer, Ile16 (I16), Leu17 (L17), Trp20 (W20) from one monomer and Trp50 (W50), Leu51 (L51), and Ile54 (I54) from the other, form a hydrophobic core which is protected from solvent by the aliphatic portions of Arg13 (R13) and Arg24 (R24). There is also a hydrogen bond between the sidechain of Glu45 (E45) and the indole nitrogen of Trp20 (W20) (O-N distance 2.8 Å).

In the crystal, each dimer associates with three other dimers to form a doughnut-like octamer (Figure 5). The octameric complex forms a pseudo-centered (C222) cell. The octamer is widely open with a central "hole", 50 Å in diameter. The open structure of the octamer is reminiscent of several other proteins, including Proliferating cell nuclear antigen (PCNA), in which the hole that is formed is believed to encircle DNA (Talluru, S.R. *et al.*, *Cell*, 79:1233-1243 (1994)). It is this octameric structure which is the translational repeating unit in the crystal (Figure 5). The dimer-dimer interface is a four-helix bundle formed across the crystallographic two-fold axis. The interface of the two dimers consists of hydrophobic residues in region A of the coiled-coil domain Leu17 (L17) and Val21 (V21) but also includes residues C-terminal to the coiled-coil

domain, region C, between residues 50 to 60 Trp50 (W50), Ile54 (I54), Ile57 (I57) and Ile58 (I58) (Figure 6). Thus, hydrophobic residue from both helices pack in the interface, essentially extending the hydrophobic core mentioned above. Trp50 is involved in both the formation of the dimer as well as the octamer. Formation of the octamer buries an additional 800\AA^2 of surface area per monomer, which means that approximately 40% of the total surface area of each monomer is buried. The 50 Å diameter hole framed by the four dimers is lined with basic sidechains (Figure 7a and b). The hole is large enough to accommodate an RNA molecule. Residues Lys26 (K26) and Lys38 (K38) which had been modeled in as alanine were changed to lysine for this calculation. The electrostatic surface was calculated using GRASP (Nicholls, A. Columbia University, New York, NY (1992)), and rendered using RASTER3D (Merritt, E. A. & Murphy, M.E.P., *Acta. Cryst. D.* 50:869-873 (1994)).

Table 1: Data Collection Statistics

	Native*	S22C Pt
Spacegroup	P2 ₁ 2 ₁ 2	P2 ₁ 2 ₁ 2
Unit cell (a,b,c)	109.2, 85.3, 29.4	110.3, 86.3, 29.6
Temperature of data collection (°C)	-160	-165
5 Resolution (Å)	15-1.73	86-2.5
Number of reflections	221, 286	44, 362
Number of unique reflections	28, 279	10, 013
Completeness [†] (%)	94 (35)	97
I/σ [†]	51 (7)	6.0
10 Multiplicity	7.8	4.4
R _{sym} ^{†‡§} (%)	4.2 (18)	6.7 [14.0]
R _{iso} [¶] (%)	-	30.5
R _{cullis} [#]	-	0.62 (0.52)
R _{cullis anom} [¥]	-	0.84
15 Phasing power**	-	2.2 (1.7)

* Data are from two crystals.

[†]Numbers in parentheses represent values in the highest resolutions shell.

[‡]R_{sym} = $\sum_{(h,k,l)} |I_{(h,k,l)} - \langle I_{(h,k,l)} \rangle| / \sum_{(h,k,l)} \langle I_{(h,k,l)} \rangle$, where $\langle I_{(h,k,l)} \rangle$ represents the sigma weighted average intensity of symmetry-equivalent reflections.

20 [§]The number in square brackets represents R_{anom} = $\sum | \langle I^+_{(h,k,l)} \rangle - \langle I^-_{(h,k,l)} \rangle | / \sum (\langle I^+_{(h,k,l)} \rangle + \langle I^-_{(h,k,l)} \rangle)$, where $\langle I^{\pm}_{(h,k,l)} \rangle$ represents the statistically weighted average intensity of symmetry-equivalent reflections.

[¶]R_{iso} = $\sum_{(h,k,l)} |F_{PH} - F_P| / \sum (F_P)$.

25 [#]R_{cullis} = $\sum_{(h,k,l)} | |F_{PH}| - |F_P + F_H| | / \sum_{(h,k,l)} |F_{PH} - F_P|$; number in parentheses represents R_{cullis} for centric reflections.

[¥]R_{cullis anom} = $\sum_{(h,k,l)} | |F_{PH+} - F_{PH-}|_{obsvd} - |F_{PH+} - F_{PH-}|_{calc} | / \sum_{(h,k,l)} |F_{PH+} - F_{PH-}|_{obsvd}$

**Phasing power = $\langle |F_H| / | |F_{PH}| - |F_P + F_H| | \rangle$; number in parentheses is the power for centric reflections.

Table 2: Refinement Statistics	
Resolution range (Å)	15-1.8
R_{working}^* (%)	22.5
R_{free}^\dagger (%)	27.0
5 Non-hydrogen protein atoms	1785
Solvent atoms	114
Rms from ideal geometry	
bond lengths (Å)	0.007
bond angles (°)	1.0
10 dihedral angles (°)	16.9
impropers (°)	0.59
Average B factor overall (Å ²)	29.3
mainchain	22.5
sidechain	32.4
15 solvent	34.7

* $R_{\text{working}} = \sum_{(h,k,l)} (|F_o| - |F_c|) / \sum (F_o)$, for a working set composed of 90% of the data.

† $R_{\text{free}} = \sum_{(h,k,l)} (|F_o| - |F_c|) / \sum (F_o)$, for a test set composed of 10% of the data selected randomly.

EXAMPLE 4: MASS SPECTROMETRY

20 MATERIALS AND METHODS:

r-HDAg-S was prepared as described above. The samples for mass spectrometry were prepared as follows: the r-HDAg-S was dialyzed overnight against water. Cross-linked protein was prepared by the addition of 5 µl of 0.5% glutaraldehyde to 40 µl of rHDAg-S for 5 minutes, and quenched by the addition of 5 µl
 25 of 1M ammonium acetate. Mass spectrometry was performed in the BCMP Biopolymer facility on a Perspective Biosystems Voyager-DE mass spectrometer.

RESULTS

Previous studies have suggested that both the peptide and natural HDAg derived from infected liver form multimers in solution (Wang, J.G. & Lemon S.M. *J. Virol*, 67:446-454 (1993)), (Rozzelle, J.E., Jr. *et al. Proc. Natl. Sci. USA*, 92:382-386 (1995)).

- 5 In order to investigate the significance of the octamer formed by the peptide, MALDI-TOF mass spectrometry was used to determine the mass of monomeric and oligomeric forms of recombinant small delta antigen, r-HDAg-S. The uncrosslinked protein has a mass of 2,1832 Da (Figure 8A), which is the correct mass within 0.01% of the amino acid sequence of the American strain of the small delta antigen (HDAg-S)
- 10 (Genbank accession # -M28267) minus the first methionine residue. The primary species of the cross-linked rHDAg-S had a mass of 176,282 Da (Figure 8B). The M+1 and M+2 peaks of the octamer were the only significant peaks in the spectrum. The ratio of the masses of the cross-linked species to the monomer is 8.1:1.

- While this invention has been particularly shown and described with references
- 15 to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the claims.

CLAIMS

What is claimed is:

1. A fusion molecule comprising HDAG and at least one binding moiety.
2. The fusion molecule of Claim 1 wherein the binding moiety is selected from the group consisting of an antigen, an antibody, a single chain antibody, a ligand, a receptor, an enzyme, a ligand interaction peptide, a chemical, an effector, an oligonucleotide, a signal amplification peptide, an enhancer recognition protein, a promoter binding protein, a label, a growth factor, a cytokine, a nuclease, a small organic molecule, a test substance, a cytotoxic agent, a substrate, a solid substrate, a drug or a fragment thereof.
3. The fusion molecule of Claim 1 which comprises two binding moieties which are binding partners.
4. The fusion molecule of Claim 1 which is a fusion protein.
5. The fusion molecule of Claim 1 wherein the HDAG and the binding moiety are chemically linked.
6. The fusion molecule of Claim 1 wherein the HDAG and the binding moiety are expressed as a single unit.
7. A coiled-coil oligomer comprising at least two fusion molecules of Claim 1.
8. The coiled-coil oligomer of Claim 7 which is an octamer.

9. The coiled-coil oligomer of Claim 7 wherein two fusion molecules are the same.
10. The coiled-coil oligomer of Claim 7 wherein two fusion molecules are different.
11. An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:
 - 5 a) a nucleotide sequence depicted in Figure 9, nucleotides 37 - 150 of Figure 9, nucleotides 37 - 186 of Figure 9, Figure 10, nucleotides 1421 - 1566 of Figure 10, nucleotides 1457 - 1566 of Figure 10, Figure 15 and Figure 16;
 - b) a complementary strand of the sequence of a);
 - 10 c) DNA sequences that hybridize to the sequence of a) or b); and
 - d) RNA sequences transcribed from the sequences of a), b) or c), or a fragment or mutation thereof, which encodes a coiled-coil oligomer.
12. An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of:
 - 15 a) a nucleotide sequence encoding a polypeptide comprising an amino acid sequence depicted in a row of Figure 1, amino acids 12 - 48 of a row of Figure 1, the top row of Figure 3C, Figure 9, amino acids 12 - 48 of a row of Figure 9, Figure 10, amino acids 12 - 88 of Figure 10, Figure 11 and Figure 17;
 - 20 b) the complementary strand of the sequence of a);
 - c) RNA sequences transcribed from the sequences of a) or b), or a fragment or mutation thereof, which encodes a coiled-coil oligomer.
13. An isolated nucleic acid molecule encoding a fusion molecule of Claim 1.

14. A fusion gene comprising a nucleic acid molecule of Claim 11 operably linked to a nucleic acid molecule encoding a heterologous peptide.
15. A fusion gene comprising a nucleic acid molecule of Claim 12 operably linked to a nucleic acid molecule encoding a heterologous peptide.
- 5 16. A recombinant polypeptide comprising an amino acid sequence encoded by a nucleic acid molecule of Claim 11.
- 10 17. An isolated and purified molecule comprising a polypeptide having an amino acid sequence selected from the group consisting of an amino acid sequence depicted in a row of Figure 1, amino acids 12 - 48 of a row of Figure 1, amino acids 12 - 60 of a row of Figure 1, the top row of Figure 3C, Figure 9, amino acids 12 - 48 of Figure 9, amino acids 12 - 60 of Figure 9, Figure 10, Figure 11 and Figure 17, or a fragment or derivative thereof which forms a coiled-coil oligomer.
- 15 18. A derivative of an HDAG peptide wherein a serine residue is substituted with cysteine.
19. An isolated and purified molecule comprising a polypeptide comprising an amino acid sequence of amino acids 12 - 88 of HDAG, or a fragment or derivative thereof which forms a coiled-coil oligomer and nuclear localization signal.
- 20 20. A polypeptide encoded by a fusion gene of Claim 14.
21. A polypeptide encoded by a fusion gene of Claim 15.

22. A vector comprising a nucleic acid molecule which encodes a subunit of an HDAG coiled-coil octamer.
23. A vector comprising a nucleic acid molecule of Claim 11.
24. A vector comprising a nucleic acid molecule of Claim 12.
- 5 25. A vector comprising a nucleic acid molecule of Claim 13.
26. A vector comprising a nucleic acid molecule encoding a fusion molecule of Claim 1.
27. A vector comprising a nucleic acid molecule encoding HDAG and at least one multiple cloning site.
- 10 28. The vector of Claim 27 wherein at least one multiple cloning site is located 3' to the nucleic acid molecule encoding HDAG.
29. The vector of Claim 27 wherein at least one multiple coding site is located 5' to the nucleic acid molecule encoding HDAG.
30. The vector of Claim 27 wherein there are at least two multiple coding sites,
15 wherein at least one multiple coding site is located in a flanking region 3' to the nucleic acid molecule encoding HDAG and at least one multiple coding site is located in a flanking region 5' to the nucleic acid molecule encoding HDAG.
31. A vector comprising a nucleic acid molecule of Claim 11 and at least one multiple cloning site.

32. A vector comprising a nucleic acid molecule of Claim 12 and at least one multiple cloning site.
33. The vector of Claim 32 further comprising a nucleic acid molecule encoding a nuclear localization signal.
- 5 34. The vector for expression of the fusion molecule of Claim 1 wherein a first heterologous gene encodes a first binding moiety and a second heterologous gene encodes a second binding moiety.
35. A vector of Claim 27 which further comprises a nucleic acid molecule which encodes a heterologous gene.
- 10 36. A host cell which comprises a nucleic acid molecule which encodes a fusion molecule of Claim 1.
37. A host cell which comprises a nucleic acid molecule of Claim 11.
38. A host cell which comprises a nucleic acid molecule of Claim 12.
39. A method of manufacturing a host cell comprising a nucleic acid molecule
15 encoding a fusion molecule comprising HDAG and at least one binding moiety comprising introducing a vector of Claim 26 into the host cell.
40. A method of expressing a high valency display of at least one binding moiety comprising introducing into a cell a vector comprising a nucleic acid molecule encoding HDAG and a nucleic acid molecule encoding the binding moiety and

culturing the cell under conditions sufficient to permit expression of a fusion molecule comprising the binding moiety and HDAg.

41. A method of enhancing interaction between binding partners comprising contacting a fusion molecule of Claim 1 with a second binding moiety wherein the first and second moieties are binding partners.
42. A method of Claim 41 wherein the fusion molecule presents the first and second binding moieties.
43. The method of Claim 41 wherein the interaction between ligands occurs in solution, on membranes or on surfaces.
44. A method of Claim 41 wherein the fusion molecule is a subunit of a coiled-coil oligomer and the first and second moieties are bound to the oligomer.
45. The method of Claim 41 whereby fusion of a first cell and a second cell is enhanced.
46. A method for delivering molecules to a cell comprising contacting them with a fusion molecule of Claim 1.
47. The method of Claim 46 wherein the binding moiety is an oligonucleotide.
48. The method of Claim 46 wherein the oligonucleotide hybridizes to a nucleic acid molecule in the cell.

49. The method of Claim 47 wherein said fusion molecule further comprises a double-stranded nuclease.
50. The method of Claim 46 wherein the fusion molecule comprises a first binding moiety and a second binding moiety wherein the first binding moiety interacts
5 with a binding partner and the second binding moiety functions as an effector.
51. The method of Claim 50 wherein the first binding moiety interacts with a cell surface receptor and the second binding moiety can kill the cell.
52. A method of amplifying a signal in a solid phase assay comprising coupling an HDAG octamer with at least one copy of a domain which interacts with a ligand
10 and at least two copies of a label.
53. A method of Claim 52 wherein the label is selected from the group consisting of alkaline phosphatase, a radiolabel, streptadavin and green fluorescent protein.
54. The method of Claim 52 wherein the solid phase assay is an ELISA assay.
- 15 55. A method of facilitating exchange of substrates and products comprising coupling an HDAG oligomer to at least two enzymes which function in a linked pathway.
56. A method of enhancing a reaction between binding partners comprising coupling the binding partners to an HDAG oligomer.

57. A method of enhancing a reaction between two binding partners comprising coupling one binding partner to an HDAg oligomer and contacting the oligomer to a second binding partner.

OLIGOMERIZATION OF HEPATITIS DELTA ANTIGEN

ABSTRACT OF THE DISCLOSURE

This invention relates to HDAg peptides, including mutants, derivatives fragments and fusion molecules, including fusion proteins, coiled-coil oligomers,
5 nucleic acid molecules, vectors comprising HDAg nucleic acid molecules, cells comprising said molecules, methods of multivalent expression and association of binding moieties of HDAg fusion molecules, and methods of use involving the molecules.

The molecules are particularly useful as a framework for multivalent display via
10 formation of C-terminal and/or N-terminal fusion proteins or via chemical coupling to chemically reactive sidechains, e.g. cysteine residues.

[illegible]

*
D
*
D
*

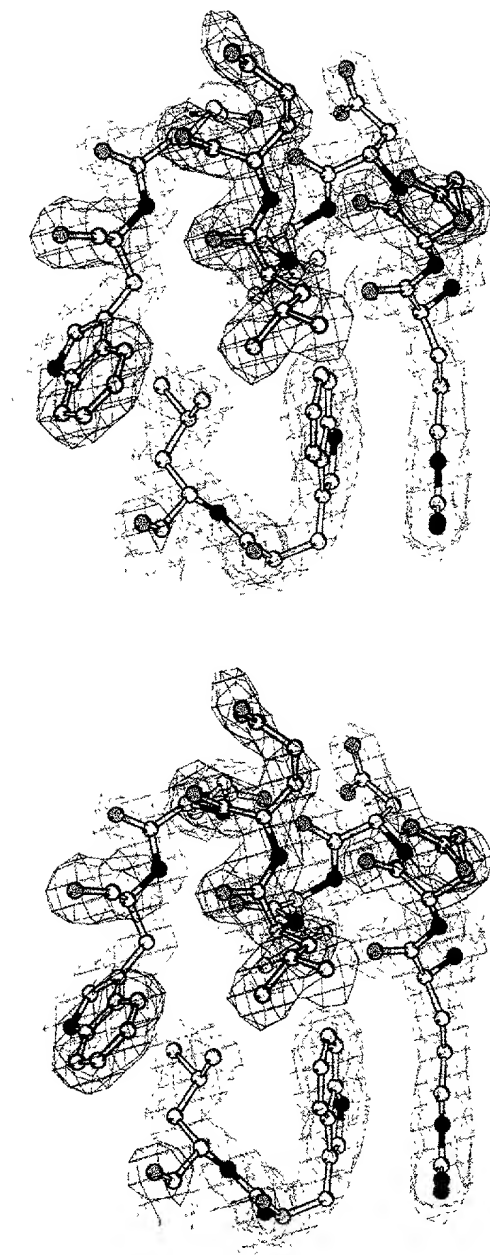
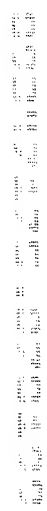


Figure 2

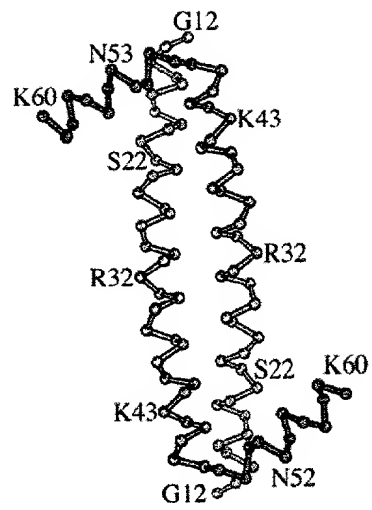
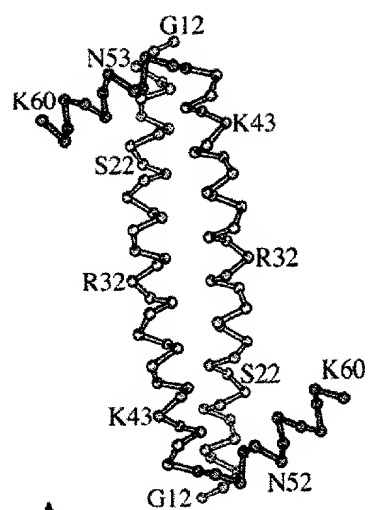


Figure 3A

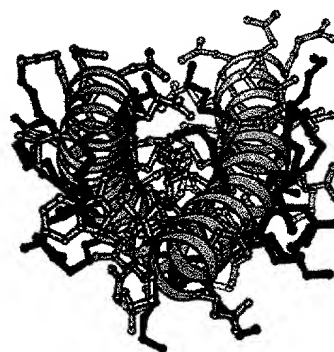
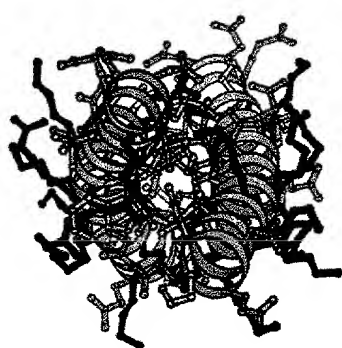


Figure 3B

B

A

12

48

gabcdefgabcdefgabcdefgabcdefgabcdefga

GREDILEQWVSGRKKLEELERDLRKLKKKIKKLEEDN

NDEELKKIKKKLKRLDRELEELKKRGSVWQELIDERG

agfedcbagfedcbagfedcbagfedcbagfedcbag

48

12

Figure 3C

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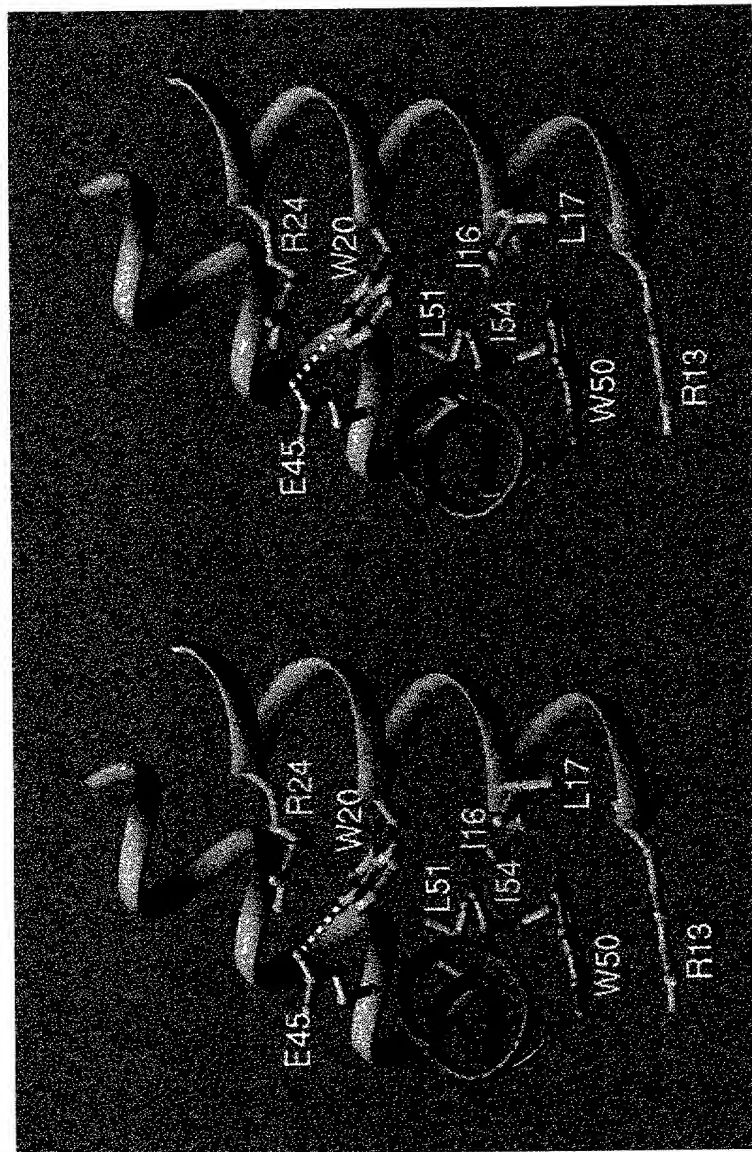


Figure 4

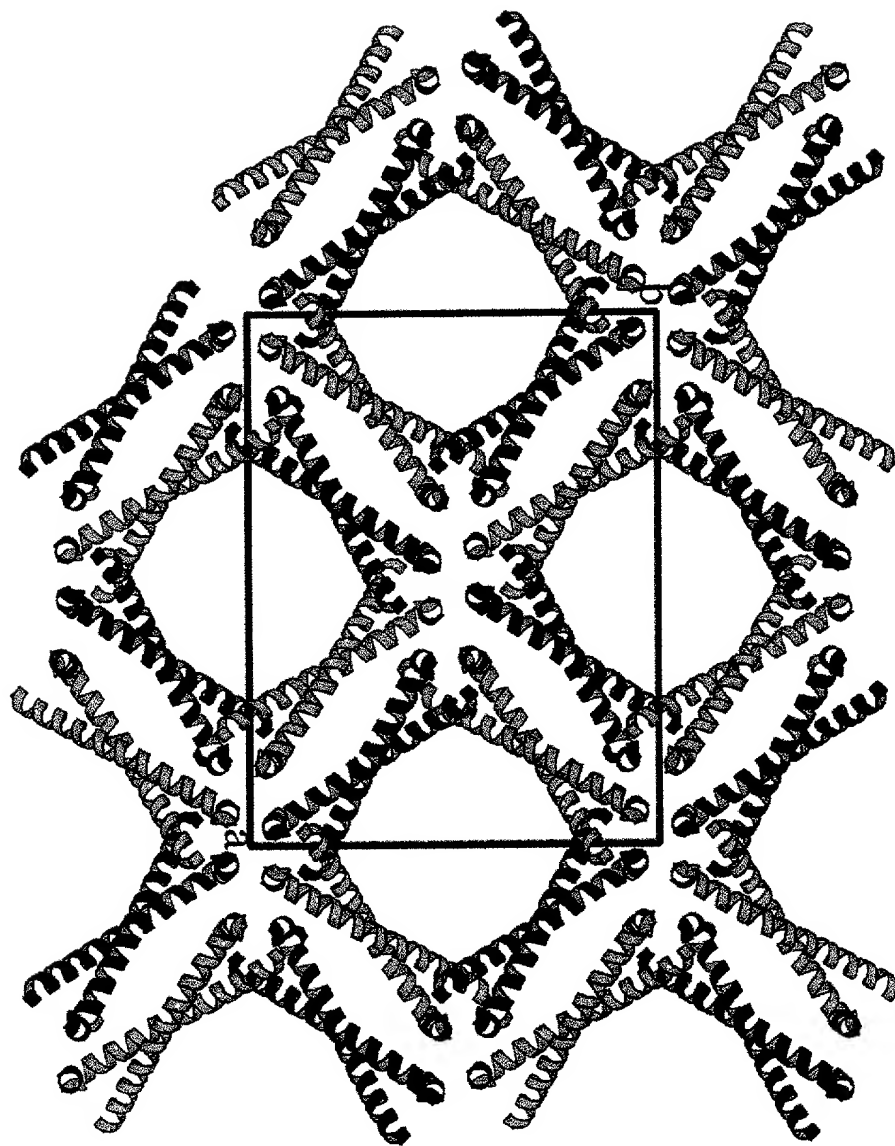


Figure 5

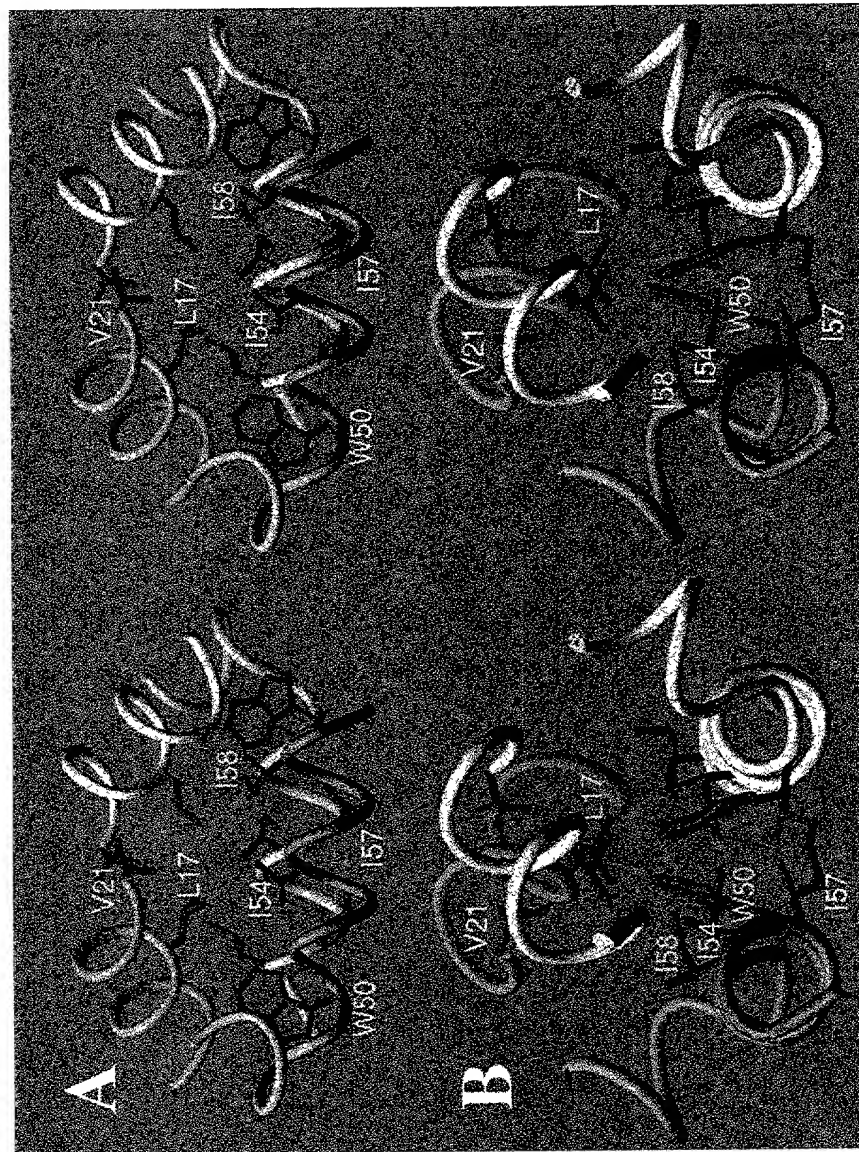


Figure 6A

Figure 6B

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LIBRARY

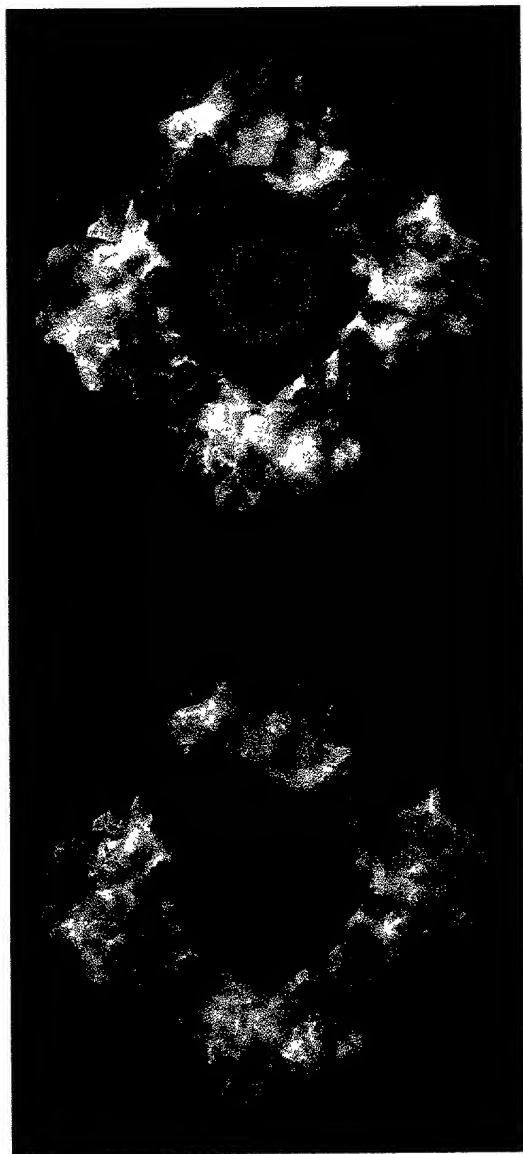


Figure 7B

Figure 7A

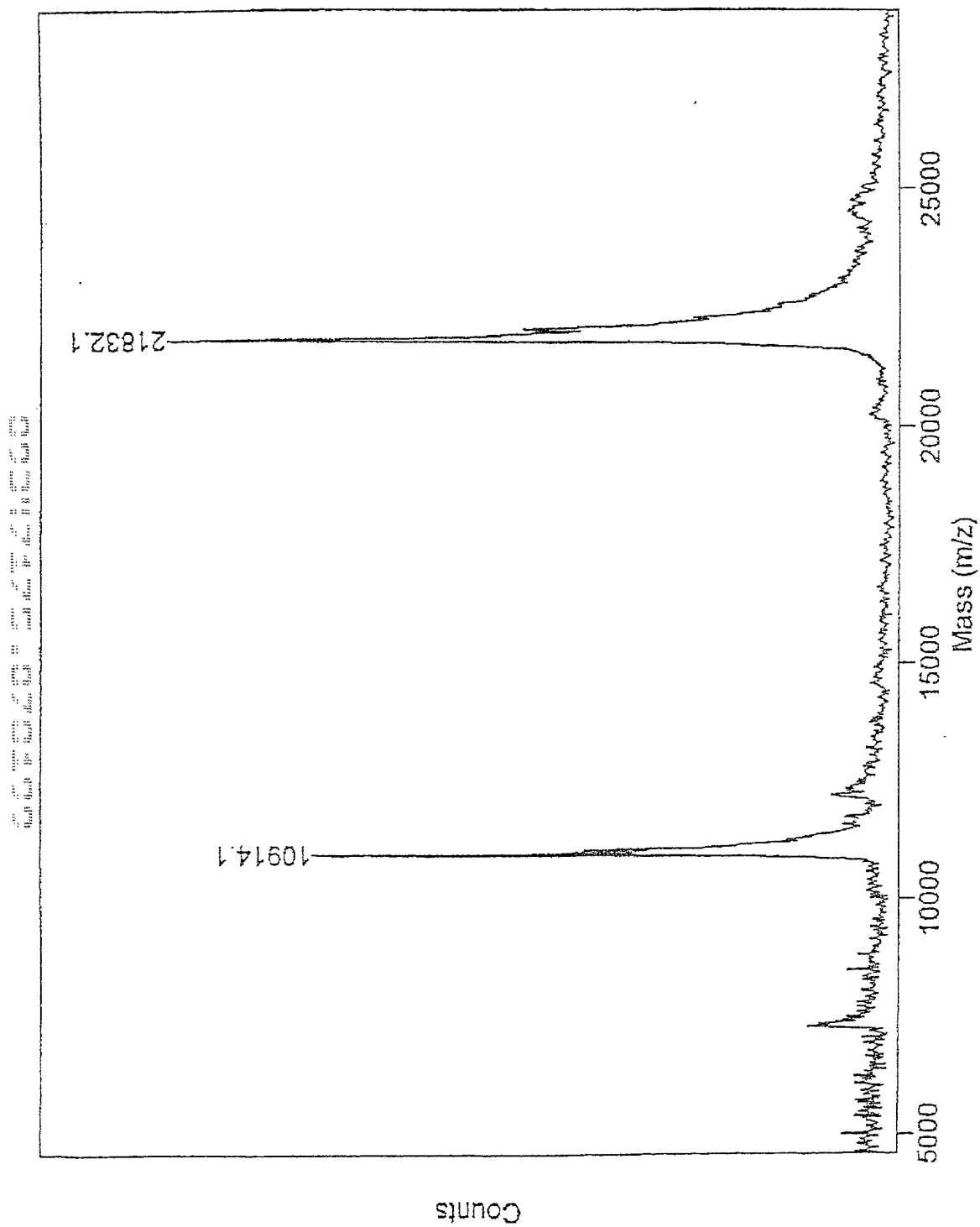


FIGURE 8A

Counts

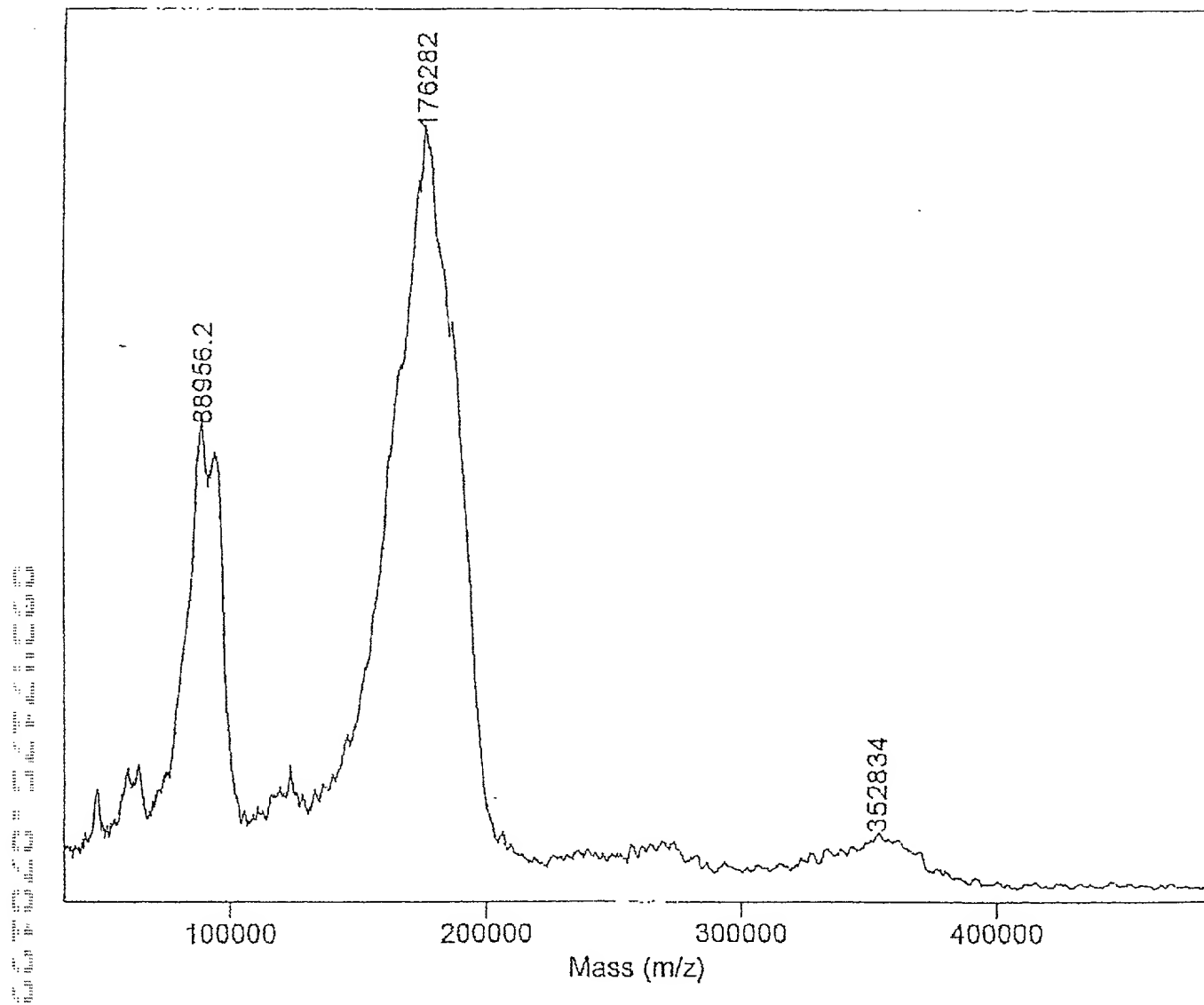


FIGURE 8B

NdeI

M S R S E R R K D R G G R E D I L E
GGGCATATGAGCCGTAGCGAAGCTCGTAAAGATCGTGGAGCCGTGAAGATATTCTGGAA
CCCGTATACTCGGCATCGCTTCAGCATTTCTAGCACCGCCGGCACTTCTATAAGACCTT

Q W V S G R K K L E E L E R D L R K L K
CAGTGGGTGAGCGGCCGTAGAAGTTAGAGGAATTGGAACGTGATCTGCGTAAACTGAAA
GTCACCCACTCGCCGGCATTCTTCAATCTCCTTAACCTTGCACTAGACGCATTTGACTTT

K K I K K L E E D N P W L G N I K G I I
AAGAAGATTAAGAACTGGAAGAAGATAACCCGTGGTTGGGTAATATTAAAGGCATTATT
TCTTCTAATTCTTTGACCTTCTTCTATTGGGCACCAACCCATTATAATTTCGGTAATA

G K K D K D G E G A P P A K K L R M D Q
GGCAAGAAAGATGAAGATGGCGAAGCGCGCCGCCGCGGAAGAACTGCGTATGGATCAG
CCGTTCTTTCTATTCTTACCGCTTCCGCGCGCGCGCCGCTTCTTTGACGCATACCTAGTC

M E I D A G P R K R P L R G G F T D K E
ATGGAATTTGATGCGGGCCCCGCTAAACGTCCGCTGCGTGGCGGCTTTACCGATAGGAA
TACCTTTAAGTACGCGCGCGCGCATTTCGAGCGGACGCGCCGGAATGGCTATTCTCTT

R Q D H R R R K A L E N K R K Q L S S G
CGTCAGGACCATCTCGTCTGTAAGCGCTGGAAACAAACGTAAACAGCTGAGCAGCGGC
CGAGTCTCTGCTAGCAGCAGCATTTCCGACCTTTTGTGTTGCACTTGTCCACTCGTCGCCG

G K S L S R E E E E E L K R L T E E D E
GGCAAACTCTCTGAGCCGTGAAGAAGAAGAAGAACTGAAACGTCTGACCGAAGAAGATGAA
CCGTTTGAAGACTCGGCACCTTCTTCTTCTTCTTGAATTTGCAGACTGGCTTCTTCTACTT

K R E R R I A G P S V G G V N P L E G G
AAACGTGAACGTCTGTTGAGGTCCATCTGTTGGTGGTGTGAACCCGCTGGAAGGCGGC
TTTGCACTTGCAGCATAACGTCCAGGTAGACAACACCACACTTGGGCGACCTTCCGCCG

S R G A P G G G F V P S M Q G V P E S P
AGCCGTGGTGCACCGGGCGGTGGCTTTGTGCCGCTATGCAAGGTGTCCAGAGAGCCCG
TCGGCACCACTGGCCCCGCCACCGAAACACGGCAGATACGTCCACAAGGTCTTTCCGGGC

F A R T G E G L D I R G S Q G F P *NcoI*
TTTGCCGCTACCGCGAAGGCCTGGATATTCTGTCGAGCCAGGGCTTTCCGTAAACCATGGCGC
AAACGGCATGGCCGCTTCCGGACCTATAAGCACCGCTCGGTCCCGAAAGGCATTGGTACCGCG

Figure 9

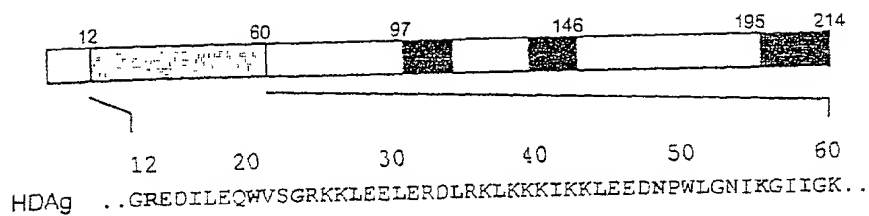


Figure 11A

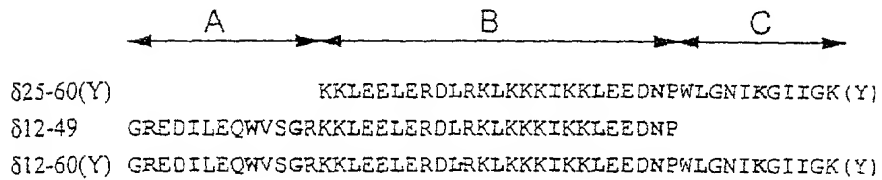


Figure 11B

Figure 12A shows a model of a protein structure, specifically a dimeric protein. The structure is composed of two subunits, each represented by a dark, textured sphere. These spheres are connected by a complex, interlocking network of smaller, lighter-colored spheres and rods, forming a central core. The overall shape is roughly circular, with the subunits positioned at opposite ends of the structure. The image is a high-contrast, black and white representation, likely a computer-generated model or a high-resolution micrograph.

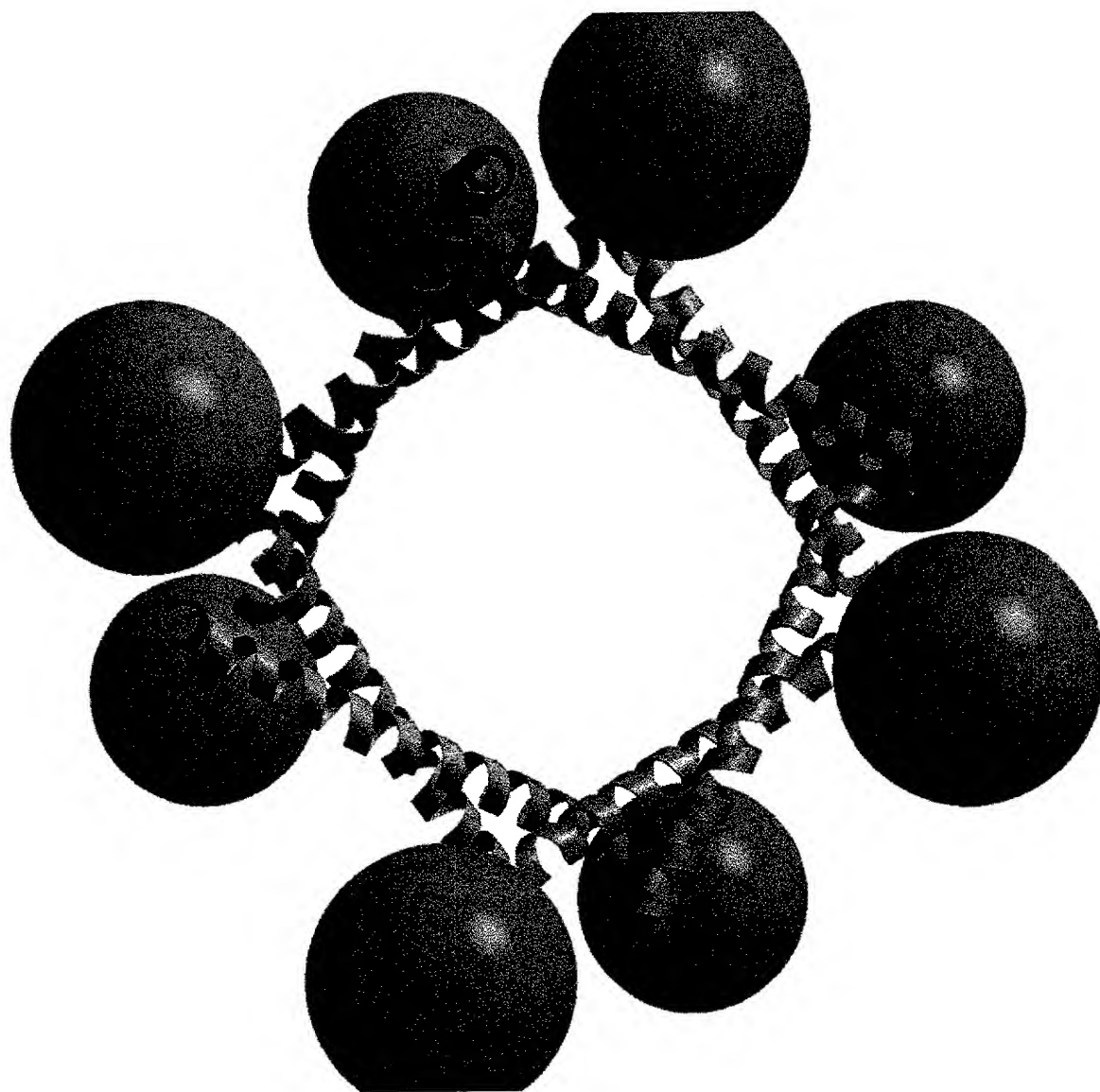


Figure 12A

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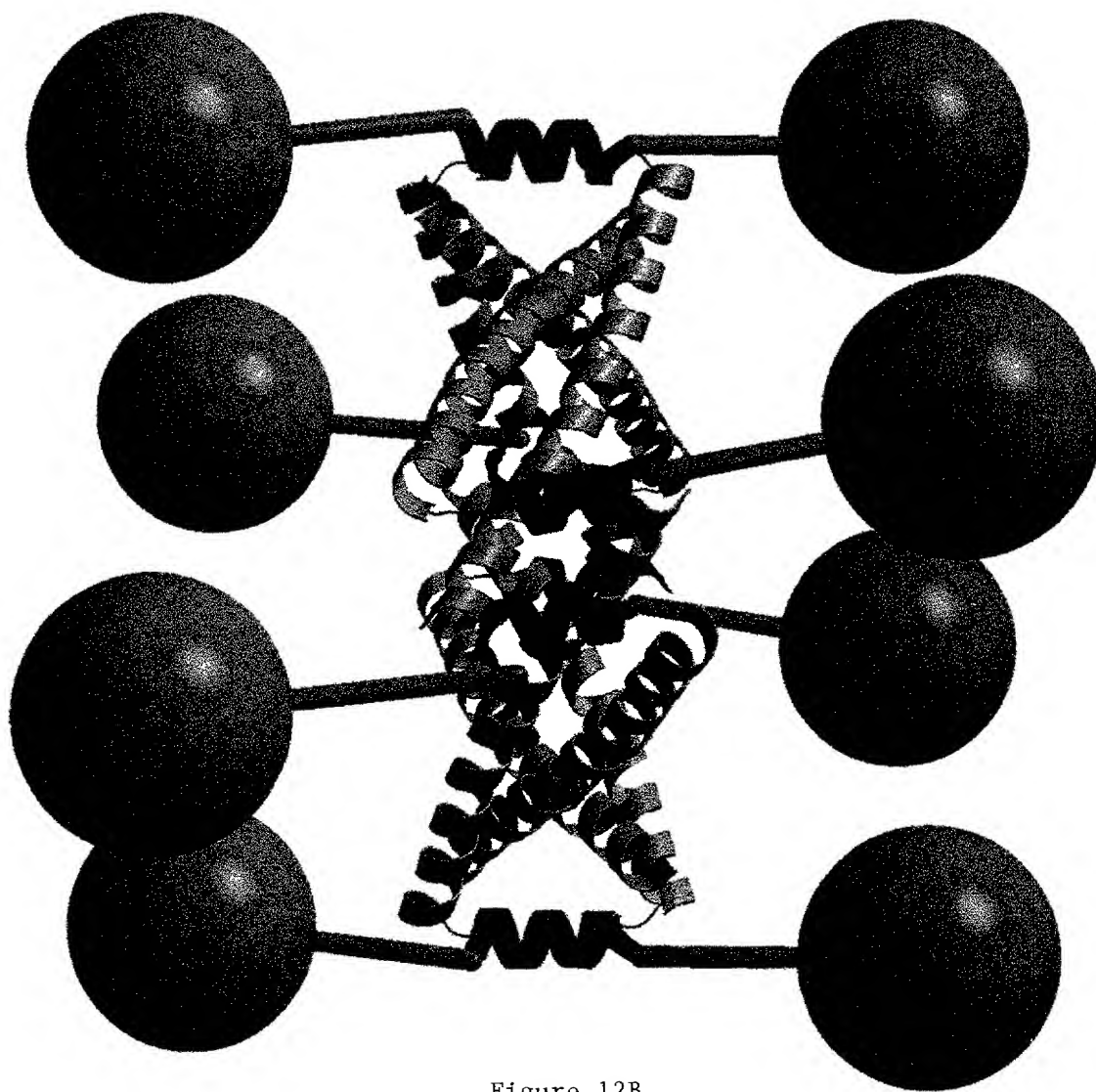


Figure 12B

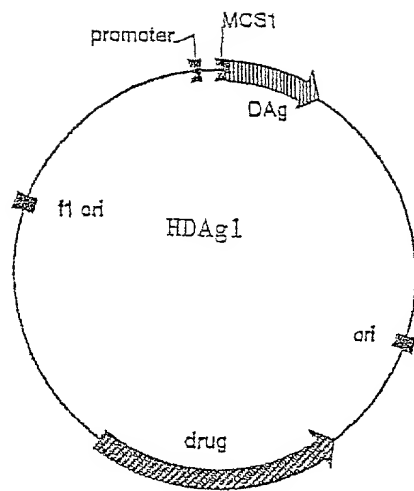


Figure 13a

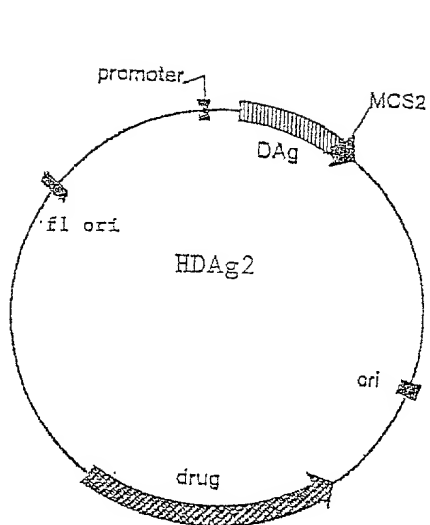


Figure 13b

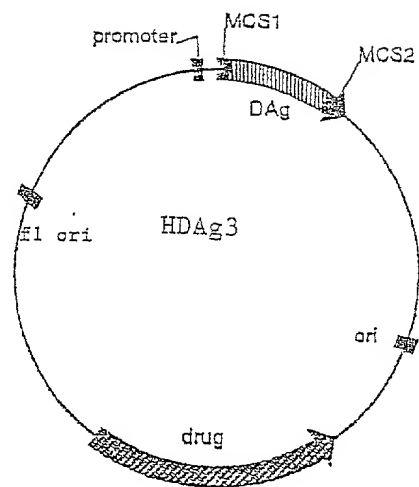


Figure 13c

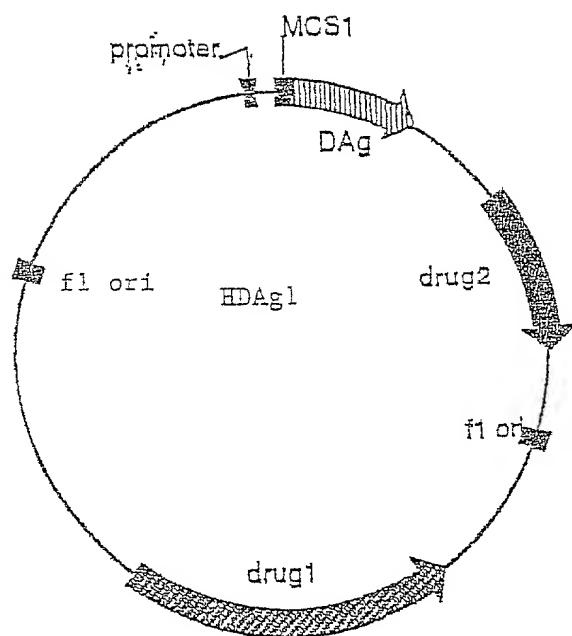


Figure 14

	1		50
synthetic ORF	ATGAGCCGta	gCGAAcGtcG tAAAGAtCGt GGcGGccGtG AAGAtATtCT	
wildtype ORF	ATGAGCCGgt	cCGAAaGaaG gAAAGAcCGc GGgGGgaGgG AAGAcATcCT	
Identity	ATGAGCCG--	-CGAA-G--G -AAAGA-CG- GG-GG--G-G AAGA-AT-CT	
	51		100
synthetic ORF	gGAaCAGTGG	GTGAGCGGcc GtAAGAAGTT AGAGGAAtTg GAacGtGAtC	
wildtype ORF	cGAgCAGTGG	GTGAGCGGaa GaAAGAAGTT AGAGGAACtC GAgGaGAGAcC	
Identity	-GA-CAGTGG	GTGAGCGG-- G-AAGAAGTT AGAGGAA-T- GA--G-GA-C	
	101		155
synthetic ORF	TgCGtAAaCT	gAAaAAGAAg ATtAAGAAAC' TgGAaGAAGA tAACCCgTGG	
wildtype ORF	TcCGgAAgtT	aAagAAGAAa ATcAAGAAAC TaGAgGAAGA cAATCCcTGG	
Identity	T-CG-AA--T	-AA-AAGAA- AT-AAGAAAC T-GA-GAAGA -AA-CC-TGG	
	151		205
synthetic ORF	tTGGGtAAtA	TtAAAGGcAT tATtGGcAAG AAaGATAAaG ATGGcGAaGG	
wildtype ORF	cTGGGaaAcA	TcAAAGGaaT aATcGGaAAG AAgGATAAgG ATGGaGAGgG	
Identity	-TGGG-AA-A	T-AAAGG-AT -AT-GG-AAG AA-GATAA-G ATGG-GA-GG	
	201		255
synthetic ORF	cGCgCCgCCG	GCGAAGAAaC TgCGtATGGA tCAGATGGaa ATtGAtGCgG	
wildtype ORF	gGCaCCcCCG	GCGAAGAAgC TcCGgATGGA cCAGATGGAg ATaGAcGCcG	
Identity	-GC-CC-CCG	GCGAAGAA-C T-CG-ATGGA -CAGATGGA- AT-GA-GC-G	
	251		305
synthetic ORF	GcCCGcGtAA	acGtCCgCTg cGtGGcGGcT TtACCGAtAA GGAacGtCAG	
wildtype ORF	GaCCtaGgAA	gaGgCCTcTc aGgGGaGGaT TcACCGAcAA GGAgGgCAG	
Identity	G-CC--G-AA	--G-CC-CT- -G-GG-GG-T T-ACCGA-AA GGA--G-CAG	
	301		355
synthetic ORF	GACcAtCGtC	GtcGtAAaGC gCTgGAaAAC AAacGtAAaC AGCTgagcag	
wildtype ORF	GATcAcCGaC	GaaGgAAGGC cCTcGAgAAC AAgAgGAAgC AGCTatcgtc	
Identity	GA-CA-CG-C	G--G-AA-GC -CT-GA-AAC AA--G-AA-C AGCT-----	
	351		405
synthetic ORF	cGGcGGcAAa	tctCTgAGCc GtGAaGAaGA AGAaGAACtG AAacGtcTGA	
wildtype ORF	gGGgGGaAAg	agcCTcAGCa GgGAgGAgGA AGAgGAACtT AAgAgGgtTGA	
Identity	-GG-GG-AA-	---CT-AGC- G-GA-GA-GA AGA-GAACT- AA--G--TGA	
	401		455
synthetic ORF	CCGAaGAAGA	tGAaAAAcGt GAAcGtcGtA TtGCaGGtCC aTctGTTGGt	
wildtype ORF	CCGAgGAAGA	cGAgAAAAgG GAAaGaaGaA TaGCCcGGcCC gTCgGTTGGg	
Identity	CCGA-GAAGA	-GA-AAA-G- GAA-G--G-A T-GC-GG-CC -TC-GTTGG-	
	451		505
synthetic ORF	GGTGTGAACC	CgCTgGAAGG cGGcagccGt GGtGCaCCgG GcGGtGGCTT	
wildtype ORF	GGTGTGAACC	CcCTcGAAGG tGGatcgaGg GGaGCgCCcG GgGGcGGCTT	
Identity	GGTGTGAACC	C-CT-GAAGG -GG-----G- GG-GC-CC-G G-GG-GGCTT	
	501		555
synthetic ORF	tGTgCCgtct	ATGCAAGGtG TtCCaGAaag CCCgTTtGCg CGtACCGGcG	
wildtype ORF	cGTcCCcagc	ATGCAAGGAg TcCCgGagtc CCCcTTcGct CGgACCGGgG	
Identity	-GT-CC----	ATGCAAGG-G T-CC-GA--- CCC-TT-GC- CG-ACCGG-G	
	551		603
synthetic ORF	AaGGcCTGGA	tATtCtGtGgC AGCCAGGGcT TtCCgTaaac cATggcgc	
wildtype ORF	AgGGaCTGGA	cATAaGgGGA AGCCAGGGaT TcCCaTggga tATactct	
Identity	A-GG-CTGGA	-AT--G-GG- AGCCAGGG-T T-CC-T---- -AT-----	

Figure 15

1 GGGCATATGA GCCGTAGCGA ACGTCGTAAA GATCGTGGCG GCCGTGAAGA
51 TATTCTGGAA CAGTGGGTGA GCGGCCGTAA GAAGTTAGAG GAATTGGAAC
101 GTGATCTGCG TAAACTGAAA AAGAAGATTA AGAACTGGA AGAAGATAAC
151 CCGTGGTTGG GTAATATTAA AGGCATTATT GGCAAGAAAG ATAAAGATGG
201 CGAAGGCGCG CCGCCGGCGA AGAACTGCG TATGGATCAG ATGGAAATTG
251 ATGCGGGCCC GCGTAAACGT CCGCTGCGTG GCGGCTTTAC CGATAAGGAA
301 CGTCAGGACC ATCGTCGTCT TAAAGCGCTG GAAAACAAAC GTAAACAGCT
351 GAGCAGCGGC GGCAAATCTC TGAGCCGTGA AGAAGAAGAA GAACTGAAAC
401 GTCTGACCGA AGAAGATGAA AAACGTGAAC GTCGTATTGC AGGTCCATCT
451 GTTGGTGGTG TGAACCCGCT GGAAGGCGGC AGCCGTGGTG CACCGGGCGG
501 TGGCTTTGTG CCGTCTATGC AAGGTGTTCC AGAAAGCCCC TTTGCGCGTA
551 CCGGCGAAGG CCTGGATATT CGTGGCAGCC AGGGCTTTCC GTAAACCATG
601 GCGC

Figure 16

	1		48
wildtype HDag-S	MSRSERRK DRGGREDILE QVSGRKKLE ELERDLRKLK KIKKLEEDN		
pR5DV5 plasmid	MSRSERRK DRGGREDILE QVSGRKKLE ELERDLRKLK KIKKLEEDN		
Identity	MSRSERRK DRGGREDILE QVSGRKKLE ELERDLRKLK KIKKLEEDN		
	49		98
wildtype HDag-S	PWLGNIKGII GKKDKDGE GA PPAKKLRMDQ MEIDAGPRKR PLRGGFTDKE		
pR5DV5 plasmid	PWLGNIKGII GKKDKDGE GA PPAKKLRMDQ MEIDAGPRKR PLRGGFTDKE		
Identity	PWLGNIKGII GKKDKDGE GA PPAKKLRMDQ MEIDAGPRKR PLRGGFTDKE		
	99		148
wildtype HDag-S	RQDHRRRKAL ENKRKQLSSG GKSLSR EEEE ELKRLTEEDE KRERRIAGPS		
pR5DV5 plasmid	RQDHRRRKAL ENKRKQLSSG GKSLSR EEEE ELKRLTEEDE KRERRIAGPS		
Identity	RQDHRRRKAL ENKRKQLSSG GKSLSR EEEE ELKRLTEEDE KRERRIAGPS		
	149		195
wildtype HDag-S	VGGVNPLEGG SRGAPGGGFV PSMQGVPE SP FARTGEGLDI RGSQGFP		
pR5DV5 plasmid	VGGVNPLEGG SRGAPGGGFV PSMQGVPE SP FARTGEGLDI RGSQGFP		
Identity	VGGVNPLEGG SRGAPGGGFV PSMQGVPE SP FARTGEGLDI RGSQGFP		

Figure 17

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primer1

GGGCATATGAGCCGTAGCGAACGTCGTAAAGATCGTGGCGGCCGTGAAGATA
TTCTGGAACAGTGGGTGAGCGGCCGTAAGAAGTTAGAGGAA

primer2

ATATTACCCAACCACGGGTTATCTTCTTCCAGTTTCTTAATCTTCTTTTT
CAGTTTACGCAGATCACGTTCCAATTCCTCTAACTTCTTACGGCC

primer3

TAACCCGTGGTTGGGTAATATTAAAGGCATTATTGGCAAGAAAGATAAAG
ATGGCGAAGGCGCGCCGCGGCGAAGAACTGCGTATGGATCAG

primer4

GATGGTCCTGACGTTCTTTATCGGTAAAGCCGCCACGCAGCGGACGTTTA
CGCGGGCCCGCATCAATTTCCATCTGATCCATACGCAGTTTCTT

primer5

ATAAGGAACGTCAGGACCATCGTCGTCGTAAAGCGCTGGAAAACAAACGT
AAACAGCTGAGCAGCGCGCGCAAATCTCTGAGCCGTGAAGAAG

primer6

CAACAGATGGACCTGCAATACGACGTTTACGTTTTTCATCTTCTTCGGTC
AGACGTTTCAGTTCTTCTTCTTCTTTCACGGCTCAGAGAT

primer7

TATTGCAGGTCCATCTGTTGGTGGTGTGAACCCGCTGGAAGGCGGCAGCC
GTGGCGCGCCGGGCGGCGGCTTTGTGCCGTCTATGCAAGGTGTTCCAGAA
A

primer8

GCGCCATGGTTTACGGAAAGCCCTGGCTGCCACGAATATCCAGGCCTTCG
CCGGTACGCGCAAACGGGCTTTCTGGAACACCTTGCATAG

primer9

GGGCATATGAGCCGTAGCGA

primer10

GCGCCATGGTTTACGGAAAG

Figure 18